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Development of sucrose-complexed lipase to improve its transesterification activity and stability in organic solvents

Shota Kajiwara [a], Ryosuke Yamada [a,*], Hideki Mori [b], Masayuki Hara [b], Hiroyasu Ogino [a]

[a] Department of Chemical Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan
[b] Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570, Japan

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ABSTRACT

A commercial lipase from *Pseudomonas fluorescens* was complexed with sucrose by freeze-drying to make the microenvironment around the lipase more hydrophilic in organic solvents, and then the stability and transesterification activity of sucrose-complexed lipases in organic solvents was examined. A lipase solution with 5 mM Tris-HCl buffer (pH 9.0) containing 1% (w/v) sucrose was found to be optimal for preparation of the sucrose-complexed lipase by freeze-drying. By complexed with sucrose, not only the lipase retains a higher proportion of its activity after incubation in 100% of *n*-decane, *n*-hexane, 1-octanol, 1-pentanol, or 1-propanol at 30 °C for 24 h, but the lipase also showed a higher transesterification activity in 100% (v/v) *n*-hexane and *n*-hexane containing 50% (v/v) of *n*-octane, 1-octanol, 1-pentanol, 1-propanol, or acetone. Using the simple and easy-to-use procedure, the sucrose-complexed lipase which can retain its activity in organic solvents was obtained.

1. Introduction

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyzes a wide range of bioconversions, including hydrolysis, esterification, transesterification, and aminolysis. Thus, lipases are used to modify lipids, pharmaceutical agents, agricultural chemicals, perfumes, and to synthesize functional materials [1,2]. Rates of lipase-catalyzed reactions typically can be increased in the presence of organic solvents, in which substrates have better solubility than in water. Furthermore, in the presence of organic solvents, lipases can catalyze not only ester hydrolysis, but also ester synthesis and transesterification [3]. However, the inactivation of lipases caused by organic solvents often becomes a problem. Therefore, stable lipases in the presence of organic solvents are required.

Until now, easy and versatile techniques for endowing an enzyme with an organic solvent stability have not been established. For example, screening microorganisms that produce organic solvent-stable enzymes requires an enormous amount of time and effort [4]. Furthermore, in the case of using protein engineering techniques, such as site-directed mutagenesis [5] and directed evolution [6], to improve the enzyme organic solvent stability is difficult because versatile techniques have not been established and it also requires an enormous amount of time and effort. Although enzyme immobilization techniques have often been used to retain enzymatic activity in organic solvents, they are laborious owing to the complicated process, as well as the problem of enzyme inactivation during immobilization [7,8]. To bind an amphiphilic polymer, such as polyethylene glycol, to the enzyme incurs the problem of enzyme deactivation during the covalent binding reaction [9–11]. Moreover, the method of coating the enzyme with lipids or surfactants has the risk of harming the human body by contamination of leached lipids and surfactants into the products in food industry [12–15].

The hydrophilicity around enzyme molecules is related to the enzyme organic solvent stability [16]. In the presence of organic solvents, if the hydrophilicity around enzyme is low, the enzyme will be easily inactivated. This is because water molecules around the enzyme, which form the hydration shell and maintain the native conformation of the enzyme, are displaced easily by direct contacts with the molecules of the organic solvents (Fig. 1a). However, if the hydrophilicity around enzyme is high, tightly bound water molecules create a favorable aqueous microenvironment around the enzyme and the frequency of direct contacts between the organic solvent molecules and the enzyme molecules would be decreased, and as a result, the enzyme can maintain its activity in the presence of the organic solvents.

Sugar, such as sucrose, is hydrophilic molecule which have many –OH groups to coordinate water molecules [17]. Furthermore, sugar

* Corresponding author.
E-mail address: yamada@chemeng.osakafu-u.ac.jp (R. Yamada).

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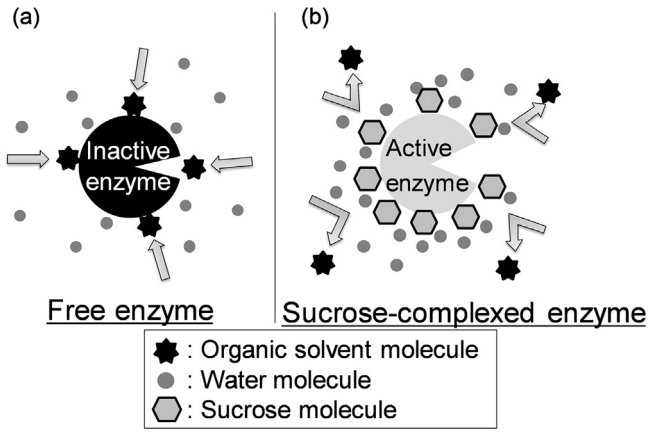

Fig. 1. Schematic illustration of (a) enzyme inactivation and (b) stabilization by sucrose complexation in the presence of organic solvents.

is harmless, and thus, favorable to use in the food industry. Freeze-drying removes water from a frozen sample containing protein by sublimation and desorption. However, some water molecules were deposited on the dried sample after freeze-drying and they keep the flexibility of the protein that is necessary for its activity [18]. The technique of freeze-drying is widely used for the preparation of various enzymes or the proteins for pharmaceutical development in order to get a solid and active form of the proteins [18,19]. Thus, if the complexing of sucrose and enzyme is achieved by freeze-drying, tightly bound water molecules surrounding the sucrose will create a favorable aqueous microenvironment around the enzyme and decrease the frequency of direct contacts between the organic solvent molecules and the enzyme molecules (Fig. 1b). As a result, the enzyme can retain its activity in the presence of organic solvents. This would be a promising and versatile technique to avoid enzyme inactivation in organic solvents.

In this study, a commercial lipase from *Pseudomonas fluorescens* was complexed with sucrose by freeze-drying and the stability and transesterification activity of sucrose-complexed lipases in organic solvents was examined.

2. Materials and methods

2.1. Materials

Lipase AK "Amano" derived from *P. fluorescens* was purchased from Amano Enzyme (Nagoya, Japan). Sodium acetate, sodium dihydrogen phosphate, tris (hydroxymethyl) aminomethane (Tris), glycine, ammonium iron (III) sulfate, vinyl stearate, acetone, *n*-hexane, *n*-octane, *n*-decane, 1-propanol, 1-pentanol, 1-octanol, and 4 Å molecular sieves were from Nacalai Tesque (Kyoto, Japan). 3-metyl-2-benzothiazolinonehydrazone hydrochloride hydrate (MBTH) was from Tokyo Chemical Industry (Tokyo, Japan). All organic solvents were stored over 4 Å molecular sieves for at least 24 h.

2.2. Partial purification of the lipase

Lipase AK was partially purified by acetone precipitation. Seventy-five milligrams of Lipase AK were solubilized in 30 mL of 5 mM Tris-HCl buffer (pH 7.5) or sodium phosphate buffer (pH 9.0). After addition of 120 mL of cold acetone at −20 °C to the enzyme solution to make a final concentration of 80% (v/v), the resultant insoluble fraction was collected by centrifugation at 20,000 × *g* at 4 °C for 30 min. The supernatant was removed and the precipitate was air-dried at room temperature.

2.3. Preparation of sucrose-free lipase and sucrose-complexed lipase

Sucrose-free lipase was prepared as follows: partial purified lipase was dissolved in 45 mL of 5 mM sodium acetate buffer (pH 4.0, 5.0, 6.0), sodium phosphate buffer (pH 6.0, 7.0, 8.0), Tris-HCl buffer (pH 8.0, 9.0), or glycine-NaOH buffer (pH 9.0, 10.0). Then, a 1-mL aliquot of the solution was freeze-dried. Sucrose-complexed lipase was prepared as follows: partial purified lipase was dissolved in 45 mL of 5 mM Tris-HCl buffer (pH 9.0) containing 0.1%–5% (w/v) sucrose. Then, a 1-mL aliquot of the solution was freeze-dried.

2.4. Stability of sucrose-free lipase and sucrose-complexed lipase in organic solvents

Sucrose-free lipase and sucrose-complexed lipase were suspended in 0.5 mL of various organic solvents (*n*-decane, *n*-octane, *n*-hexane, 1-octanol, 1-pentanol, 1-propanol, or acetone) and the suspension was incubated at 30 °C with a shaking speed of 150 rpm for 1 h or 24 h. After incubation, the transesterification activity was measured.

2.5. Measurement of transesterification activity of the lipase

The transesterification activity of the lipase was measured using vinyl stearate and 1-pentanol as substrates, which was a modification to the method by Zheng et al. [20]. The reaction was carried out in a sealed tube. Sucrose-free lipase or sucrose-complexed lipase was suspended in 0.5 mL of an organic solvent (*n*-decane, *n*-octane, *n*-hexane, 1-octanol, 1-pentanol, 1-propanol, or acetone). Then, 0.5 mL of 100 mM vinyl stearate in *n*-hexane and 10 μL of 100% (v/v) 1-pentanol were added to the suspension and incubated at 30 °C with a shaking speed of 150 rpm for 10 min. This mixture was centrifuged at 20,000 × *g* at 4 °C for 30 min, and 10 μL of the supernatant was diluted 1:100 in deionized water. Four hundred microliters of the dilution and 400 μL of 0.1% (w/v) MBTH solution were mixed together. After incubation at 30 °C for 10 min, 160 μL of 1% (w/v) ammonium iron (III) sulfate solution (in 0.1 M HCl) was added. Then, the mixture was incubated at 30 °C for 30 min. Following this, colorimetric measurements were carried out at 598 nm by a Multiskan^{TM} GO Microplate Spectrophotometer (Thermo Fisher Scientific, Bremen, Germany). The control experiment was carried out without the addition of enzyme. One international unit (I.U.) of the transesterification activity was defined as the amount of enzyme that produced 1 μmol of vinyl alcohol per min at 30 °C.

3. Results and discussion

3.1. Effects of pH during freeze-drying on the transesterification activity

To evaluate the effects of pH and buffering agents during the freeze-drying process on the transesterification activity of Lipase AK, the partial purified lipase was dissolved in buffers of various pH and freeze-dried, and the transesterification activity was measured. As shown in Fig. 2, when using acetate buffer (pH 4.0, 5.0, 6.0), sodium phosphate buffer (pH 6.0, 7.0, 8.0), or glycine-NaOH buffer (pH 9.0, 10.0), the sucrose-free lipase showed a similar transesterification activity. On the other hand, when using the Tris-HCl buffer (pH 8.0, 9.0), the sucrose-free lipase had a higher activity at pH 9.0 than at pH 8.0. The freeze-dried lipase using Tris-HCl buffer (pH 9.0) showed the highest transesterification activity among all the freeze-dried lipases prepared in this study.

It is reported that when a protein was freeze-dried, amino groups, carboxyl groups, and phenyl groups contained in the protein maintained their charge state before drying, and thus, the pH