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An improved method of lipase preparation incorporating both solvent treatment and immobilization onto matrix

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Abstract

A simple and effective preparation of lipases for use in organic solvents is hereby proposed. Lipases in aqueous solution were treated with isopropanol, immediately followed by immobilization onto a commercially available macroporous resin CRBO2 (crosslinked polystyrene with *N*-methylglucamine as a functional group). The dual modification of lipases by (1) isopropanol treatment and (2) immobilization improved the activity and stability of lipases more significantly than either of the two treatments alone. The degree of lipase activation was dependent on isopropanol–buffer (v/v) ratio and the source of lipase used. Among the lipases tested, *Rhizopus oryzae* lipase was more significantly activated. The maximum specific activity of *R. oryzae* lipase after dual modification was 94.9 mmol h^{-1} g^{-1}, which was, respectively, 3.3-, 2.5- and 1.5-fold of untreated free, untreated immobilized and treated free lipases. The conformations of the treated and untreated free lipases were investigated by circular dichroism (CD) measurement. Changes in the far- and near-UV CD spectra of lipase indicate that lipase activation is accompanied by changes in secondary and tertiary structures of lipases. The increase in negative molar elipticity at 222 nm suggests that the α-helical content of lipase increase after pretreatment.

Keywords: Lipase; Immobilization; Isopropanol treatment; Enzyme activity; Adsorption; Macroporous resin

1. Introduction

Enzymes are synthetically useful catalysts in nonaqueous organic solvent. Although numerous enzymes are catalytically active in organic solvent, a serious problem is the much lower activity of enzyme in anhydrous media than in water [1]. Thus, it is crucial to activate enzymes in organic solvent. A various ways of lipase activation in organic media have been reported. These include the use of enzyme powder suspended in either microaqueous organic solvent or biphasic system [2]. Furthermore, lipases have been hosted in reverse micelle [3] and in some cases, chemically or physically modified [4]. Recently, the enhancements in enzyme activity, stability and enantioselectivity of lipase by treatment with polar organic solvent have been reported [5]. The treatment with polar organic solvent changes the lipase conformation from the less hydrophobic-closed form (active site is covered by lid) to more hydrophobic open form (active site is opened), favoring the binding of hydrophobic substrate to lipase [5,6].

Another area of interest in enzyme technology is the immobilization of enzymes onto a solid support to improve their usefulness. Yu et al. [7] have improved the activity of *Candida rugosa* lipase by 1.5-fold by immobilizing it on a macroporous resin, CRBO2 (crosslinked polystyrene with *N*-methylglucamine as a functional group). Many immobilization methods were studied including adsorption [8], covalent attachment [9] and entrapment in matrix of porous support materials [10]. However, the enzymes employed in previous studies were not treated with organic solvent and have the same limitations as untreated lipase. Thus, the objective of this study to develop a suitable method of lipase preparation that incorporates both solvent pretreatment and immobilization. The active site of pretreated lipase is expected to remain open in immobilization form to give a higher catalytic activity

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in organic solvents than the lipase without organic solvent treatment.

Here, we report that the pretreatment of lipases in aqueous solution by placing them in contact with isopropanol, immediately followed by immobilization onto macroporous resin (CRBO2) via adsorption significantly improved the activities of lipases of different origins when compared with isopropanol treatment or immobilization alone. The motivations of using isopropanol as the pretreatment solvent are that there are some published data regarding enzyme activation using this solvent [5,6,11] than those using other polar solvents (e.g. ethanol, methanol and acetone), and that isopropanol has higher effectivity for increasing enzyme activity than others solvents [5,12]. The circular dichroism (CD) spectra of *Rhizopus oryzae* lipase suggest that changes in both secondary and tertiary structure accompanied lipase activation induced by isopropanol pretreatment.

2. Materials and methods

2.1. Materials

Lipases (EC 3.1.1.3) from the *Porcine pancreas* and *R. oryzae* were purchased from Fluka (Switzerland), and lipases (EC 3.1.1.3) from *C. rugosa* and *Mucor javanicus* were from Sigma (USA) and Amano Enzyme Inc. (Japan), respectively. Macroporous adsorptive resin DIAION CRBO2 was from Mitsubishi Chemical Corporation (Japan). Lauric acid and 1-dodecanol were, respectively, from Sigma (USA) and Fluka (Switzerland). Isopropanol was purchased from J.T. Baker, USA. All chemicals were of analytical grade and used as received, unless otherwise specified.

2.2. Methods

2.2.1. Treatment of DIAION CRBO2

The resin (CRBO2) was washed with deionized water and isopropanol subsequently until neutrality. The resin was collected by filtration and dried in desiccator under vacuum.

2.2.2. Preparation of protein stock solution

The lipase solution (4 mg/ml) prepared by dissolving powder lipase in 5 mM phosphate buffer (pH 7) was centrifuged at 5000 rpm to remove insoluble impurities. The upper layer was collected and used as a protein stock solution for the subsequent experiments. The protein content in the stock solution was measured from the UV absorbance at 280 nm using bovine serum albumin as the standard [7].

2.2.3. Solvent treatment followed by immobilization

Experimental procedure for the dual modification of lipases by treatment with isopropanol followed by immediate immobilization onto a resin is as follows. A protein stock solution (28 ml) was mixed with 12 ml of isopropanol and stirred at 4 °C for 3 h as the activation of lipase reached a plateau at 3 h of pretreatment time (Fig. 1). CRBO2 (3 g) was then added to the solution and the mixture was incubated at 4 °C without stirring for 24 h. The CRBO2 with immobilized lipase was collected by filtration and dried by lyophilization. The free lipase concentration in filtrate was measured from UV absorbance at 280 nm. The lipase concentration in filtrate was subtracted from that in isopropanol–water solution before adding CRBO2, and based on the difference in lipase concentration the amount of lipase that adsorbed onto CRBO2 was calculated. The yields of lipase immobilization with and without isopropanol treatment after 24 h are 35 and 40%, respectively. No precipitation was observed in the solutions throughout the immobilization.

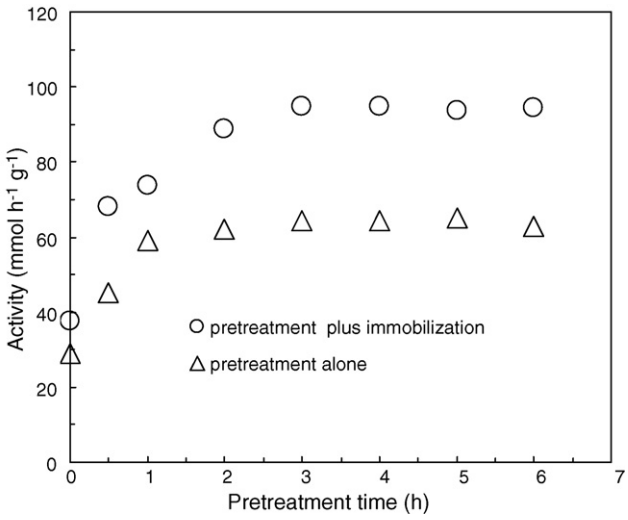

Fig. 1. Effect of pretreatment time on activity of *R. oryzae* lipase. Activity assay conditions: total reaction volume 10 ml, $C_{\text{lauric acid}} = 0.1$ M, $C_{\text{1-dodecanol}} = 0.1$ M, water 0.1 ml, temperature 25 °C and reaction time 3 h, immobilized or free lipases equivalent to protein 0.2–0.4 mg ml^{-1}.

2.2.4. Solvent treatment only

The protein stock solution (28 ml) was mixed with 12 ml of isopropanol, stirred at 4 °C for 3 h, frozen and lyophilized to obtain powder lipase.

2.2.5. Immobilization of lipase without solvent treatment

The experimental procedure was the same as that for solvent treatment followed by immobilization (described above), except for the fact that the solvent (12 ml) was replaced with phosphate buffer (pH 7).

2.2.6. Determination of lipase activity

The reaction mixture (10 ml) consisted of lauric acid (0.1 M), 1-dodecanol (0.1 M) and free or immobilized lipases (protein equivalent to 0.2–0.4 mg ml^{-1}) in isooctane with a small amount of water (0.1 ml) was incubated at 25 °C for 3 h with continuous shacking at 250 rpm. It was observed that the amount of acid consumed was linearly dependent on protein concentration up to 1.25 mg ml^{-1}. A sample (5 ml) was withdrawn and mixed with 10 ml of ethanol-acetone (1:1, v/v) to stop the reaction. The remaining acid in the sample was determined by titration with 0.05 M NaOH. Specific lipase activity (mmol h^{-1} g^{-1}) was defined as the amount of acid consumed per hour per gram protein.