

Poly(ethylene glycol)-lipase complex highly active in ionic liquids

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INTRODUCTION

A number of studies on enzymatic reactions have been carried out in organic media. These reactions have several benefits compared with conventional organic synthesis, including high enantioselectivity, substrate specificity and low energy consumption. There are, however, several drawbacks in using organic solvents. Volatile organic solvents are often flammable and toxic to the environment and the human body. Moreover, the enantioselectivity of enzymatic reactions in organic solvents has not yet satisfied industrial needs.

In the last decade, ambient temperature ionic liquids have attracted interest as alternative reaction solvents (Huddleston *et al.*, 1998; Cull *et al.*, 2000; Erbeldinger *et al.*, 2000). Ionic liquids, formally called molten salts, are liquids composed entirely of ions. They are liquid over the broad temperature range and have almost no volatility. By modification of the counter cation and anion, their properties (e.g. hydrophobicity) can be tuned in many ways. Moreover, ionic liquids are simple and inexpensive to manufacture and easy to recycle. Recent studies on chemical reactions in ionic liquids have focused on transformations using transition-metal catalysis (Chauvin *et al.*, 1995). Recently, several groups have used ionic liquids for enzymatic reactions. Erbeldinger *et al.* (2000) reported that (Z)-aspartame synthesis could be catalyzed by thermolysin in an ionic liquid, and Itoh *et al.* (2001) and Schofer *et al.* (2001) reported lipase-catalyzed high-enantioselective acylation in ionic liquids. Interestingly, enzymatic reactions in ionic liquids tend to have high enantioselectivity. These studies demonstrate the high utility of ionic liquids as a novel medium for enzymatic reactions. In terms of industrial application, a high enzymatic activity is required in ionic liquids. However, there are few reports on the activation of enzymes for catalysis in ionic liquids.

So far many enzyme-activation methods have been developed for enzyme-catalyzed reactions in organic solvents. Murakami and Hirata (1999) developed a simple activation method involving the formation of a poly(ethylene glycol)-enzyme complex. In the present study, we have applied this method to lipases and studied alcoholysis catalyzed by the PEG-lipase complex in ionic liquids. We have demonstrated that the formation of a PEG-lipase complex is effective for the use of lipases in ionic liquids.

MATERIALS AND METHOD

Lipase AK (from *Pseudomonas fluorescens*), lipase AY (from *Candida rugosa*), lipase F (from *Rhizopus oryzae*), lipase M (from *Mucor javanicus*), and lipase PS (from *Pseudomonas cepacia*) were kindly provided by Amano Enzyme Inc (Nagoya, Japan). The protein contents of the lipases were determined by the biuret method (Gornall AG *et al.*, 1949), using bovine serum albumin as the standard. The protein contents of lipases AK, AY, F, M and PS were 33.2, 27.2, 59.4, 29.0, 3.9 %, respectively. Cinnamic acid, benzyl cinnamate, vinyl cinnamate and benzyl alcohol

were purchased from Tokyo Chemical Industry, Japan. 1-Methylimidazole, 1-chlorobutane, 1-chlorohexane and 1-chlorooctane were obtained from Aldrich. All other chemicals were purchased from Wako Pure Chemicals Ltd., Japan.

Synthesis of ionic liquids

The synthesis of ionic liquids as described by Cull *et al.* (1998) and Huddleston *et al.* (1998) was modified. 1-Methylimidazole (103 g, 1.25 mol) and 1-chlorobutane (139 g, 1.5 mol), 1-chlorohexane (189 g, 1.5 mol) or 1-chlorooctane (223 g, 1.5 mol) were combined in a flask containing a magnetic stirrer bar. The flask was connected to a reflux condenser and a positive nitrogen flow was maintained over the contents. The flask was immersed in a silicone oil bath at 70°C. The reaction was continued under these conditions for 72 h. Excess chloroalkane (upper phase) was decanted and the residual chloroalkane was removed under reduced pressure and heating (110°C). The product was washed three times with 250 mL of ethyl acetate. Residual ethyl acetate was removed under reduced pressure.

1-Butyl-3-Methylimidazolium Hexafluorophosphate [Bmim][PF₆], 1-hexyl-3-methylimidazolium hexafluorophosphate [Hmin][PF₆] and 1-Octyl-3-Methylimidazolium Hexafluorophosphate [Omim] [PF₆]

Ammonium hexafluorophosphate (200 g, 1.3 mol) in 0.5 L of water and [Bmim][Cl] (184 g, 1.0 mol), [Hmin][Cl] (208 g, 1.0 mol) or [Omim][Cl] (231 g, 1.0 mol) in 0.5 L of water were mixed, stirred vigorously for 2 h, and incubated at room temperature for 1 night. The two phases formed were allowed to separate, and the upper aqueous phase was decanted. The lower organic phase was washed twice with 500 mL of water and saturated sodium bicarbonate solution, respectively. Ionic liquids were extracted from the mixture using dichloromethane (250 mL), and the organic phase was removed under reduced pressure at 110°C. The remaining water in the ionic liquids was removed by lyophilization over 1 day. The synthesized ionic liquids were confirmed by elemental analysis and FT-IR (FT-IR 8300, Shimadzu) (Chun S. *et al.*, 2001).

Preparation of poly(ethylene glycol)-lipase complexes

The preparation of a PEG-lipase complex as described by Murakami and Hirata (1999) was modified. Each lipase (10 mg) and poly(ethylene glycol) 20,000 (PEG) were dissolved in phosphate buffer (5 mL, pH 7.0). The typical molar ratio (PEG/lipase) was 10. For effective coating of the lipase surface (Kamiya *et al.*, 1997), toluene (13 mL) was added to the lipase solution and homogenized at 20,000 rpm for 3 min using an ULTRA-TURRAX T25 (Junke & Kunkel) in an ice bath to prepare water-in-oil emulsions. The w/o emulsions were immediately frozen in liquid nitrogen, followed by lyophilization for 24 h using a freeze-drying machine. White powders were obtained as the PEG-lipase complexes. As a control, the native lipase was lyophilized from the phosphate buffer solution.

Enzymatic reaction in ionic liquids

Alcoholysis between vinyl cinnamate (10 mmol/L) and benzyl alcohol (75 mmol/L) to produce benzyl cinnamate was conducted in the ionic liquids using native lyophilized lipases or PEG-lipase complexes at 60°C. In advance, water was added to the ionic liquids (typically 0.25 % (v/v)). Benzyl cinnamate and cinnamic acid production were monitored at 254 nm using an HPLC system (JASCO 2000 series) on a 4.6×250 mm column packed with 5 μ m ODS.

RESULTS AND DISCUSSION

In nonaqueous enzymology, the dispersion of enzymes in the reaction medium is one of the essential factors for enzyme activation. Most pure organic solvents cannot dissolve native enzymes. Murakami and Hirata (1999) developed a method to overcome this problem which involves the formation of a poly(ethylene glycol)-enzyme complex (PEG-enzyme complex). They proposed that the enzyme was surrounded by PEG molecules, rendering it sufficiently hydrophobic for dissolution in organic solvents. We have applied this method to lipase PS and investigated the enzymatic performance in an ionic liquid. In the ionic liquid ([Hmin][PF6]), this PEG-lipase complex was not dissolved at all but was well-dispersed.

We investigated the alcoholysis between vinyl cinnamate and benzyl alcohol catalyzed by lyophilized native lipase PS or the PEG-lipase PS complex in [Hmin][PF6]. The PEG-lipase complex effectively catalyzed the alcoholysis reaction, whereas the lyophilized native lipase PS performed the same reaction very slowly. The alcoholysis reaction involves two reaction steps, acylation of the lipase and esterification of benzyl alcohol. The side reaction (hydrolysis) of the alcoholysis produces cinnamic acid as the byproduct. The production rate of cinnamic acid by the PEG-lipase complex was also faster than that of the lyophilized native lipase. The PEG-lipase complex significantly activated the alcoholysis in the ionic liquid along with hydrolysis. The lipase activation induced by the formation of the PEG-lipase complex can be explained by the following two factors. The first factor is the high dispersion of the PEG-lipase complex in the ionic liquid. The lyophilized lipase was not dispersed well in [Hmin][PF6], but the PEG-lipase was well dispersed (data not shown). The second factor is an oil-water interfacial activation involving a conformational change in the lipase molecule. Mingarro *et al* (1995) reported that the surface of micelles allows lipases to be highly active in nonaqueous solvents. Maruyama *et al.* (2000) also demonstrated that a hydrocarbon-water interface induces lipase activation for a nonaqueous reaction. In the present study, the improvement in the dispersion and the toluene-water interface would contribute to the lipase activation for alcoholysis.

The effect of the PEG/lipase ratio on the alcoholysis activity was also investigated in detail. Assuming that the proteins in the native lipase PS were only the lipase, the number of moles of lipase could be calculated. The initial reaction rate of alcoholysis was influenced by the PEG/lipase ratio, in agreement with the previous report (Murakami and Hirata, 1999). An approximately 10-fold amount of PEG introduced the highest activity to the lipase. At higher PEG/lipase ratios, the excess amount of PEG may interrupt the interfacial activation of the lipase at the toluene-water interface during the preparation of the PEG-lipase complex and, furthermore, cover the surface of the lipase molecule preventing substrate access to the active site of the lipase. Therefore, the higher PEG/lipase ratios resulted in low lipase activity.

Alcoholysis catalyzed by the PEG-lipase complex was studied in three different ionic liquids and in a solvent-free system (benzyl alcohol). The activities obtained are summarized in Table 1. The activity of the PEG-lipase complex depended strongly on the solvent tested. Ionic liquids with a longer alkyl chain conferred relatively high lipase activity. This result agrees with the report that a polar organic

solvent is not suitable for enzymatic reactions (Zaks and Klibanov, 1985). The PEG-lipase complex exhibited low activity in the solvent-free system (benzyl alcohol). Chulalaksananukul *et al.* (1990) reported that lipase-catalyzed esterification in an organic solvent was inhibited by alcohol. The low lipase activity in benzyl alcohol is also considered to be due to alcohol inhibition.

Several lipases of diverse origin were studied in [Omin][PF6]. The formation of the PEG-lipase complex improved the alcoholysis activity for all lipases and, among the lipases tested, the PEG-lipase PS complex exhibited the highest activity. The differences in activation among lipases may be related to the solvent tolerance of the lipases or the dispersion of the PEG-lipase complexes.

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Table 1. Initial reaction rates of lipase-catalyzed alcoholysis in ionic liquids

Solvent	Initial reaction rate [mmol/(hr g-protein)]
[Bmin][PF6]	0.55
[Hmin][PF6]	1.03
[Omin][PF6]	1.07
Benzyl alcohol	0.15

Alcoholysis was carried out between vinyl cinnamate (10 mmol/L) and benzyl alcohol (400 mmol/L) at 40°C using lipase PS (1 mg/mL). The PEG/lipase ratio was 20 [mol/mol]. 1%(v/v) water was added to the reaction medium.