Polar Organic Solvent Added to an Aqueous Solution Changes Hydrolytic Property of Lipase

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Received January 16, 2003; Accepted April 21, 2003

For developing further uses of lipase as a biocatalyst, its hydrolytic activity toward some esters was investigated in a miscible solution composed of a buffer and a polar organic solvent. Twenty percent dimethylformamide, 35% dimethylsulfoxide, 15% 1,4-dioxane, 15% dimethoxyethane, and 2% diethoxyethane promoted the hydrolysis by a lipase from Rhizomucor miehei toward some hydrophobic substrates, 4-methylumbelliferyl oleate, 4-methylumbelliferyl palmitate, and monoolein. While hydrolysis by this lipase toward the substrates with a relatively weak hydrophobicity (4-methylumbelliferyl heptanoate and 4-methylumbelliferyl nanoate) was suppressed by these solvents. A fluorometric analysis showed that the polar organic solvent in the buffer induced some conformational change around a tryptophan residue of R. miehei lipase. In addition to the influence of the miscible solvent on the solubility of the substrates, the conformational change of the protein induced by the miscible solvent would also affect the reactive properties of the lipase. Adding a polar organic solvent to an aqueous solution will be an efficient method for changing hydrolytic performance of lipases.

Key words: lipase; hydrolysis; polar organic solvent

Although lipase is one of the digesting enzymes for lipids, its industrial importance as a catalyst has been increasing. Its resistance to organic solvents, one of the advantages of this enzyme, has permitted adoption of various solvents for the biocatalytic performance. Many esterification and ester-exchanging reactions by lipase as a catalyst have been done in various organic solvents with control of water activity.1–8) These studies showed that the yield of biosynthetic product was significantly affected by the characteristics of the reaction media used. Furthermore, the combination of more than two kinds of organic solvents has been also examined for the lipase reaction. For example, the denaturant solvents, such as dimethylsulfoxide, were added to organic solvents and the yield of the product catalyzed by lipase was improved.9–11) The industrial demands accelerated only in the aspect of the ester synthesis or ester exchange processes catalyzed by lipase.

On the other hand, attention to hydrolysis by lipase, another aspect of this enzyme, has been focused on the reaction only in aqueous solutions. Hydrolysis by lipase, which plays an important role in the lipid digestion in the metabolic pathway, is also related to a first step of lipid peroxidation. So, controlling of lipid degradation is significant for food manufacturing and for food storage. Previously, some reports showed that a non-polar organic solvent in an aqueous solution affected the hydrolytic property of lipase.7,12–14) In a heterogeneous solvent, the binding of substrate-solvent complex to the enzyme contributed to the enzyme kinetics, although the enzymatic catalysis occurred with full inherent catalytic turnover.13) So, the major effect of the solvent was to cause a dramatic change in the apparent $K_m$ values.12–14) Furthermore, the efficiency of hydrolysis toward several glycerides by microbial lipases depended on the nature of the organic solvent added to the buffer.7,14) Kinetic study of enzymes under such conditions was difficult to resolve.7)

However, little information is available on the effect of a polar organic solvent in an aqueous solution on lipase activity. Especially, the action of the miscible solvents on the hydrolytic properties of lipases has not been investigated in detail.

In this work, several polar organic solvents were introduced into the reaction media for ester hydrolysis by lipase using fluorescent substrates, 4-methylumbelliferone esters and monoglycerides. For the enzyme, a lipase from Rhizomucor miehei was used, because of its considerable resistance against the organic solvents.15) Among the examined solvents, dimethylformamide, dimethylsulfoxide, 1,4-dioxane, 1,2-dimethoxyethane, and 1,2-diethoxyethane increased the hydrolysis rate toward substrates with strong hydrophobicity. While, the relative weak hydrophobic substrates were hydrolyzed slowly by lipase in the same solvent. The causes of the changes in hydrolytic properties of lipase in the miscible solvent were discussed. Based on the previ-
ous biotechnological studies, there are only four Trp residues in the \textit{R. miehei} lipase molecule. Among them, a Trp residue on the lid of the lipase could be easily characterized by its fluorescence measurement. In this work, Trp fluorescence of this lipase was investigated in various miscible solvents, and the local conformational change of the lipase, which can be deduced from Trp fluorescence, was also considered relative to changes of hydrolysis property. These results indicate that several polar organic solvents added to the aqueous solution can be expected to be a useful method for efficient hydrolysis of the hydrophobic substrates.

Materials and Methods

Reagents. Lipase from \textit{Rhizomucor miehei} was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan) and was partially purified according to the previous method. Four 4-methylumbelliferone esters (4-methylumbelliferyl palmitate (4MUP), 4-methylumbelliferyl oleate (4MUO), 4-methylumbelliferyl nonanoate (4MUN), and 4-methylumbelliferyl heptanoate (4MUH)), were obtained from Sigma (St. Louis, MO, USA). Monoglycerides used as substrates (1-monocaprin, 2-monocaprin, 1-monocapristeryl nonanoate (4MUN), and 4-methylumbelliferyl heptanoate (4MUH)), were purchased from Sigma. 9-Bromomethyl acridine (9BMA), a fluorometric method developed previously. All methylumbelliferone ester was measured by the fluorimetric-HPLC method established previously.

Results and Discussion

Effects of polar organic solvents on hydrolysis toward 4MUO by lipase from \textit{Rhizomucor miehei}

The effects of several organic solvents on the hydrolytic activity by lipase from \textit{Rhizomucor miehei} were investigated using 4MUO as a substrate. The denaturants, DMSO and DMF, were added to the enzyme solution at defined concentrations. In examined solutions, neither precipitation nor insolubility of the lipase could be detected by spectrometric measurements. Although a large decrease in hydrolytic activity was detected at a high concentration level (more than 50\%) of each denaturant, the hydrolysis was stimulated by the addition of a small amount of DMF or DMSO, as shown in Fig. 1A. The lipase in 20\% DMF showed a 2-fold higher activity than that in 100\% buffer. In the case of DMSO, the maximum hydrolysis toward 4MUO was reached at its concentration of 35\%-40\% (Fig. 1A).

As for some cyclic ethers, hydrolysis toward 4MUO by lipase was increased up to 1.9-fold by 15\% 1,4-dioxane, compared with that in the buffer alone (Fig. 1B). However, the existence of 1,3-dioxane even at a low concentration greatly inhibited hydrolysis. The inhibitory effect of THF on hydrolysis was small compared with that of 1,3-dioxane (Fig. 1B).

Next, several non-cyclic ethers were considered to investigate their effects on hydrolytic activity by lipase. Dimethoxymethane (DMM), 1,2-dimethoxyethane (DME), 1,2-diethoxyethane (DEE), 2,2-dimethoxypropane (DMP), and anisol were added to the reaction solvent. As shown in Fig. 1C, the presence of DME or DEE at a low concentration increased hydrolytic activity by the lipase. Lipase in 2\% DEE hydrolyzed 4MUO by a factor of 1.2 more rapidly than that did in the buffer. As for DME, the hydrolytic activity in 20\% DME reached a maximum at a level of 140\% of that shown in the buffer. While, increases in the concentrations of DMM, DMP, and anisol decreased hydrolysis by lipase without activation (Fig. 1C).

Finally, the hydrolysis toward 4MUO by the lipase was measured in various concentrations of alcohols, such as methanol, ethanol, \textit{n}-propanol, and \textit{n}-butanol. Previously, it was reported that the existence of \textit{iso}-propanol affected the enantioselectivity of lipase during the esterification process. In this study, it was found that the addition of alcohol to an aqueous solution inhibited hydrolysis by the lipase, as shown in Fig. 1D. Since alcohol has the OH-group, as a functional group, might act as a product inhibitor, competing with that of the substrate in the case of the hydrolytic reaction. Alcohols with the long side chains had a stronger inhibitory effect on
The relative hydrolysis rate was measured in the buffer with various concentrations of a polar organic solvent.

These findings showed that DMF, DMSO, DME, DEE, and 1,4-dioxane at each defined concentration stimulated hydrolysis toward 4MUO by the lipase at the different levels.

**Stability of lipase from R. miehei in the aqueous solution coexisted with polar organic solvents**

Because the hydrolytic activity by the lipase from *R. miehei* was considerably accelerated by 20% DMF, 35% DMSO, 15% 1,4-dioxane, and 15% DME, the stability of this lipase in each solution was investigated. As shown in Fig. 2, the hydrolytic activity by lipase in each solution remained more than 85% of the original one, even after incubation for 7 days at 37°C. On the other hand, the residual activity of the lipase in the buffer under the same incubation conditions was more than 95% of the original. This result suggested that no serious damage would be done to the lipase molecule by these examined solutions, immediately. The resistance of this lipase to polar organic solvents indicates that this enzyme can be expected to be a useful catalyst in these solutions.
Effects of DMF on lipase hydrolysis toward various esters

In addition to 4MUO, other three 4-methylumbelliferone esters were hydrolyzed and compared with their hydrolysis rates in various solvents composed of partial organic solvents. For the enzymatic reactions, the concentrations of DME, 1,4-dioxane, DMSO and DMF were set at 15%, 15%, 35%, and 20%, respectively, where the effect of each organic solvent on hydrolysis toward 4MUO reached a maximum. As shown in Fig. 3, these miscible solvents had significantly different effects on the hydrolysis rate toward four substrates, depending on the length of its acyl side chains. In the case of 20% DMF, 4MUP, and 4MUO were hydrolyzed about twice as fast, compared with those in the buffer, and the lipase in 20% DMF hydrolyzed both 4MUN and 4MUH more slowly than that did in the buffer. The hydrolysis toward 4MUN in 20% DMF was 49% of that in the buffer. As for 4MUH, only 2.6% of the activity in the buffer remained in 20% DMF (Fig. 3). The effects of DMSO, 1,4-dioxane, and DME on lipase hydrolysis toward four 4-methylumbelliferone esters were similar to that of DMF, indicating that the mode of action of these organic solvents both on lipase and on substrates would be similar, although their degrees were different.

In these experiments, we demonstrated that the added polar organic solvents increased lipase hydrolysis toward hydrophobic substrates, but that it diminished its activity toward other substrates with weak hydrophobicity. These results indicated that these miscible solvents changed the hydrolytic property of lipase. Such manufacturing of enzymatic properties would make it possible to control the selective hydrolytic activity. For instance, the hydrolysis by the lipase from R. miehei toward 4MUP in 20% DMF was 2 fold larger than that in the buffer, and this value toward 4MUH in 20% DMF was only 2.6% of that in the buffer. Therefore, this lipase will predominantly hydrolyze 4MUP, when 4MUP and 4MUH are simultaneously incubated in 20% DMF.

Effect of polar organic solvents on the interaction between R. miehei lipase and monoglycerides

When 1-monocaprin, 2-monocaprin, 1-monoolein, and 2-monoolein were hydrolyzed in 20% DMF or 35% DMSO by this enzyme, the initial hydrolysis rates were compared with those in the buffer. The hydrolysis rates toward 1-monoglycerides were more rapid than those toward 2-monoglycerides. Both 20% DMF and 35% DMSO promoted the hydrolysis rates toward all monoglycerides examined by the lipase, compared with those in the buffer (Fig. 4). Especially, these miscible solvents gave a larger activation of hydrolysis by lipase toward two monooleins than those toward monocapris. The difference of hydrophobicity between monoolein and monocaprin might affect the degree of increase of lipase activity. In the case of 4-methylumbelliferone esters as substrates, the addition of these polar solvents to the buffer also increased hydrolysis toward the hydrophobic substrates (4MUO and 4MUP), as shown in Fig. 3. These results suggested that hydrophobicity of the substrate was concerned with the increase of the hydrolysis rate by lipase in the miscible solvents.
Effects of added polar organic solvent on lipase protein

To analyze the mechanisms of changing lipase reactivity by a polar organic solvent, a fluorescence spectrum of tryptophan residues (Trp) of lipase was traced. It is known that four Trp residues exist in the mature protein of R. miehei lipase from analysis of the primary structure.16) When the lipase protein was excited with the wavelength of 280 nm, its emission spectrum was detected in the range of wavelength from 320 nm to 380 nm. The fluorescence peak of the lipase in the buffer lay at 340 nm, as shown in Fig. 5A. The fluorescence maximum of this lipase was slightly red-shifted and the lipase protein emitted its peak at 344 nm in 15% 1,4-dioxane (Fig. 5A), 20% DMF (Fig. 5B), and 35% DMSO (Fig. 5C), in which the hydrolytic property of the lipase was affected. This observation indicated that these miscible solvents had important effects in the overall spectral distribution of Trp. The difference of Trp fluorescence spectrum of the lipase between in the buffer and in the miscible solvents meant the change of the chemical environment around Trp in the lipase molecule. Some conformational change would be indicated by the existence of a polar organic solvent, that resulted in the chemical environment diversity surrounding Trp in the lipase. Furthermore, 40% DMF and 55% DMSO, which diminished the activity of the lipase, considerably red-shifted the fluorescence maximum up to 356 nm–360 nm. Only a very little change in the fluorescence spectrum of Trp was detected in 15% 1,3-dioxane (Fig. 5A), where the hydrolytic activity was extremely inhibited. The peak top of Trp fluorescent spectrum did not red-shift even in 25% 1,3-dioxane, where the hydrolytic activity was completely lost. Its intensity became somewhat weaker in that solvent (data not shown). These observations indicated the characteristic difference of 4 nm-red-shift of the fluorescence spectrum peak of Trp shown in 15% 1,4-dioxane, 20% DMF, and 35% DMSO related to the change of hydrolytic property. These solvents induced common conformational changes in the lipase, which resulted in the definite change in chemical environment surrounding Trp in the lipase. Such conformational deviation would be one of the reasons why the hydrolytic property of the lipase was changed by the addition of organic solvents. The Trp fluorescence spectrum in 15% 1,3-dioxane was not similar to those in 40% DMF and in 55% DMSO, although all of these solvents inhibited hydrolysis by the lipase completely. The degree of the red-shift of Trp fluorescent spectrum in 40% DMF and 55% DMSO is extremely large, compared with that in 20% DMF and 35% DMSO. It suggests that the large conformational changes which reflected in the chemical environment of Trp would be induced by 40% DMF and 55% DMSO, comparing with those by 20% DMF and 35% DMSO. Such conformational deviation of lipase protein might be related to the inhibition of lipase activity. On the other hand, the Trp fluorescent spectrum in 15% 1,3-dioxane indicated that 1,3-dioxane did not affect the chemical environment of Trp, although the activity of lipase was inhibited. These are two possibilities to explain this phenomenon. One is that lipase did not interact with 1,3-dioxane and that no effect of 1,3-dioxane could be detected in the Trp fluorescent spectrum. In this case, inhibition of lipase activity would be caused by the interaction of 1,3-dioxane with the substrate. The other possibility was that 1,3-dioxane would interact with lipase at a part of the protein far from Trp, but it might have a inhibitory effect on lipase activity. Such interaction of the solvent with the lipase did not affect the Trp fluorescent spectrum. The action of 1,3-dioxane to the lipase would be completely different from those of other polar organic solvents. The study of the influence of 1,3-dioxane on lipase molecule is in progress in our laboratory.

Using lipase in a non-aqueous solution, one of the serious problems is a lower activity of lipase than that is shown in an aqueous system. For avoiding this problem, DMSO was added to a non-aqueous solution and activities of esterification by some enzymes containing lipase were increased in the previous stu-
microorganisms were detected in relation to the con-

lipase at least. The previous works reported that the organic solvent aŠected the protein structure of the the lipase shown in this study suggested that the polar change of the enzyme molecule would aŠect its cata-

ture of the lipase caused by polar organic solvents in the buffer, some speculations can be proposed based on the results of this work. One is that a polar organic solvent had a different influence on the steric structure of the lipase molecule. The conformational change of the enzyme molecule would aŠect its catalytic mechanism. The fluorometric results of Trp of the lipase shown in this study suggested that the polar organic solvent aŠected the protein structure of the lipase at least. The previous works reported that the fluorometric changes of Trp in the lipase from microorganisms were detected in relation to the con-

formational change of the lipase.21,22

As for substrates, the solubility of several hydrophobic substrates was small and they might actually be dispersed in the aqueous solution without organic solvents. The addition of a polar solvent to an aqueous solution would improve the solubility of a hydrophobic substrate. Increases in solubility and dispersion of hydrophobic substrate in a miscible solvent would be concerned with an increase of hydrolysis toward them by lipase.

Or, the contributions both of the conformational change of lipase protein and of the change of sub-

strate condition induced by a polar solvent added might modulate enzyme kinetics.

The main finding of this study was that several polar organic solvents changed the hydrolytic perform-

ance of lipase. The solvent system, under which the enzyme reaction was conducted in this study, was unique for the purpose of the investigation of lipase hydrolysis. Like as the “solvent engineering” concerned with esterification reactions by lipases is a generally accepted strategy, investigations of ‘solvent effect’ by organic solvents on the hydrolytic properties of several lipases will be a clue to develop further use of lipases as biocatalysts.

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