Note

Ester Synthesis in Various Organic Solvents by Three Kinds of Lipase Preparations Derived from *Pseudomonas fragi* 22.39 B

Toshiyuki NISHIO and Minoru KAMIMURA

Research & Development Laboratories,
Sapporo Breweries Ltd., 10 Okatohme,
Yaizu, Shizuoka 425, Japan

Received April 12, 1988

In recent years, it has been reported that lipases function as catalysts for esterification or transesterification reactions in organic solvents and the activity was influenced by the kind of solvent used as a medium.^{1~4)} Therefore, selecting the organic solvent suitable for the specific lipase preparation is important to make it have higher activity.

Previously, we reported the modification of a purified lipases from *Pseudomonas* (*Ps.*) *fragi* 22.39 B with an amphipathic polymer, polyethylene glycol (PEG).⁵⁾ This modified enzyme was soluble in organic solvents such as benzene and 1,1,1-trichloroethane, and catalyzed ester synthesis from fatty acids and alcohols in these solvents. Recently, we found that the crude lipase preparation from the culture broth of this microorganism also had ester synthetic activity in benzene and 1,1,1-trichloroethane in suspended form, while the purified enzyme preparation scarcely did. Therefore, we investigated the ester synthetic activity of these lipase preparations (crude, pure, and PEG-modified ones) derived from *Ps. fragi* 22.39 B in various organic solvents, to find the more suitable organic media and compare them with each other.

The crude lipase was obtained as follows; cold acetone was added to a filtrate of the culture broth of *Ps. fragi* 22.39 B, then precipitated enzyme was recovered by centrifugation, washed with cold acetone, and dried under vacuum at room temperature. The medium composition and culture conditions were described in our previous paper.⁶⁾ This preparation contained no other enzymes which had ester synthetic activity besides the lipase. The pure lipase was prepared as described in our previous paper⁶⁾ except for lyophilization of the enzyme which was the final step of the operation. Instead of lyophilization, the purified lipase was collected by the addition of cold acetone and dried. This purified lipase preparation was confirmed to be homogeneous on polyacrylamide gel electrophoresis. PEG-modified lipase (PEG-lipase) was prepared by the method described previously.⁵⁾ The modifier, 2,4-bis(*o*-methoxypolyethylene glycol)-6-chloro-*S*-triazine, was covalently coupled with amino groups of

the lipase molecule. This PEG-lipase was lyophilized at the final step of the operation. With respect to the some enzymatic properties in aqueous solution (*e.g.* effects of pH and temperature for hydrolysis activity), differences were not observed between the three lipases. Water contents of the three lipases were 5.4, 5.2, and 3.2% (w/w) for crude, pure, and PEG-modified, respectively, measured by the Karl-Fischer method.⁷⁾ Hydrolysis activities of the three lipases were 202, 1820, and 204 units/mg of dry weight for crude, pure, and PEG-modified, respectively, assayed at 37°C and pH 7.0 by the method of Yamada.⁸⁾ One unit of the enzyme was defined as the amount which liberated 1 μ mol equivalent of fatty acid from olive oil per minute.

Using palmitic acid and cetyl alcohol as substrates, ester synthetic activities of the three lipases were measured in various organic solvents. After adding 2,000 units of the lipase to 20 ml of organic solvent containing 2 mmol of each substrate, the mixture was incubated at 25°C with constant shaking (100 oscillations/min). At intervals of 10 min, 100 μ l of the reaction mixture was taken out and 0.2 N sulfuric acid in benzene was added to it to stop the reaction, and the synthesized ester was extracted by the method of Inada.⁹⁾ The extracted ester was colorimetrically measured by the method of Snyder,¹⁰⁾ and the reaction curve of the degree of ester synthesis was drawn. The curves obtained by the reactions in various organic solvents were straight until an hour. The initial reaction rates of the ester synthesis by the three lipases were calculated from these curves. Synthesized ester was identified by thin-layer chromatography (TLC) and infrared spectroscopy. The TLC was done with a silica gel plate (Type 60, Merck Co., Ltd.) using a solvent system of petroleum ether-diethyl ether-acetic acid (70:30:1, v/v).

Palmitic acid and cetyl alcohol were purchased from Tokyo Kasei Kogyo Co., Ltd. Organic solvents (reagent grade) used as reaction media and other reagents were obtained from Wako Pure Chemicals Co., Ltd.

Table I shows the initial reaction rates of the lipase-catalyzed esterification between palmitic acid and cetyl alcohol in different organic solvents. Water in these solvents was removed by shaking them with molecular sieves 3A (Gasukuro Kogyo Co., Ltd.), then storing them in the presence of the absorbent. The water contents of these organic solvents were 0.001~0.02% (v/v), measured by the Karl-Fischer method. Though the crude lipase was insoluble in all organic solvents used, it showed high ester synthetic activity in water-immiscible organic solvents. Especially, this enzyme preparation had higher activity in hydrocarbons such as *n*-hexane, isooctane, and cyclohexane. The PEG-lipase also showed the activity in many water-immiscible organic solvents in dissolved or suspended forms, and the activity was higher in solvents such as benzene and 1,1,1-trichloroethane. Pure lipase was insoluble in all organic solvents used, and it scarcely showed any activity in them.

It was reported that water in the enzyme molecule

TABLE I. INITIAL REACTION RATES OF THE LIPASE-CATALYZED ESTERIFICATION BETWEEN PALMITIC ACID AND CETYL ALCOHOL IN DIFFERENT ORGANIC SOLVENTS

About 2,000 units of the enzyme powder (10.0, 1.1, and 9.8 mg for crude, pure, and PEG-modified lipase, respectively) was added to 20 ml of organic solvent containing 2 mmol of palmitic acid and 2 mmol of cetyl alcohol, then the mixture was shaken at 25°C. The course of the ester synthesis was followed by a colorimetric method⁹⁾ using cetyl palmitate as a standard. Water contents of the organic solvents were 0.001~0.02 (v/v) and those of lipase preparations were 5.4, 5.2, and 3.2% (w/w) for crude, pure, and PEG-modified, respectively.

Solvent	Reaction rate ($\mu\text{mol/hr} \cdot 2,000$ units of enzyme)		
	Crude lipase	Pure lipase	PEG- lipase
Benzene	110	0	372
Toluene	152	0	204
Pyridine	0	0	0
Chloroform	78	0	28
Carbon tetrachloride	990	20	386
1,1,1-Trichloroethane	566	18	888
<i>n</i> -Hexane	1242	10	128
Cyclohexane	1426	10	140
Isooctane	1532	16	222
Diethyl ether	32	0	0
1,4-Dioxane	36	0	0
Tetrahydrofuran	6	0	0
Acetone	18	0	0
Dimethylformamide	0	0	0

(enzyme-bound water) was important for both its stability and reactivity in organic solvents, and a trace of water in the organic medium also affected the enzyme reactivity.^{1,2)} The three lipase preparations scarcely showed any activity in water-miscible organic solvents. This is probably on account of the solubilization of the enzyme-bound water into these solvents. The water contents of the three lipases and organic solvents used as reaction media were different from each other, and these differences may have influenced the reactivity of each enzyme. Therefore, we investigated the ester synthetic activity of three lipases in both dehy-

drated and water-saturated organic solvents, and compared them with each other. Benzene, 1,1,1-trichloroethane, and *n*-hexane were used as reaction media. The initial reaction rates of ester synthesis of each enzyme were more or less faster in water-saturated organic solvents than in dehydrated ones, but the tendency as to the reactivity of each enzyme in these solvents did not change.

Moreover, we investigated the stability of the three lipases in various organic solvents, by suspending the enzyme powder into each organic solvent and incubating them for 2 hr at 40°C. After a 2-hr incubation, solvents were evaporated and these enzymes were dried, then their residual activities (hydrolysis activity) were measured. The three kinds of lipases which were incubated in various water-immiscible organic solvents showed the same activities as the original ones.

From the above data, we would like to emphasize that the reactivities of lipases in organic solvents are affected by the structure of the enzyme preparation, as well as water contents of the reaction mixture and stability of the enzyme against the solvents.

Acknowledgments. The authors thank Ms. Masayo Ohashi for her technical assistance.

REFERENCES

- 1) K. Takahashi, H. Nishimura, T. Yoshimoto, M. Okada, A. Ajima, A. Matsushima, Y. Tamaura, Y. Saito and Y. Inada, *Biotechnol. Lett.*, **6**, 765 (1984).
- 2) A. Zaks and A. H. Klivanov, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 3192 (1985).
- 3) S. Osanai, *J. Jpn. Oil Chem. Soc.*, **35**, 955 (1986).
- 4) A. Makita, T. Nihira and Y. Yamada, *Tetrahedron Lett.*, **28**, 805 (1987).
- 5) T. Nishio, K. Takahashi, T. Yoshimoto, Y. Kodera, Y. Saito and Y. Inada, *Biotechnol. Lett.*, **9**, 187 (1987).
- 6) T. Nishio, T. Chikano and M. Kamimura, *Agric. Biol. Chem.*, **51**, 181 (1987).
- 7) J. Mitchell, Jr. and D. M. Smith, "Aquametry," Interscience Publishers Inc., New York, 1980.
- 8) K. Yamada, Y. Ota and H. Machida, *Nippon Nōgeikagaku Kaishi*, **36**, 860 (1962).
- 9) K. Takahashi, T. Yoshimoto, A. Ajima, Y. Tamaura and Y. Inada, *Enzyme*, **32**, 235 (1984).
- 10) F. Snyder and N. Stephens, *Biochim. Biophys. Acta*, **34**, 244 (1959).