Note

Ester Synthesis by Crude Lipase Preparation from *Pseudomonas fragi* 22.39 B in *n*-Hexane

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We confirmed that *Pseudomonas* (*Ps.*) *fragi* 22.39 B produced only one type of lipase, which is a thermostable enzyme with a molecular weight of 33,000.¹⁾ We have been studying the esterifying activity of this lipase. In the previous paper, we reported the enzymatic esterification in an aqueous reaction system using the purified lipase preparation.²⁾

Recently, we found that the crude lipase preparation exhibited the esterifying activity in a suspended form in various organic solvents, while the purified one scarcely did so.³⁾ Enzymatic esterification in a nonaqueous medium has many advantages compared with that in an aqueous one, as indicated by many investigators.^{4~6)} Therefore, it is necessary for its application to investigate the various characteristics of this lipase preparation on ester synthesis in an organic solvent.

The previous paper³⁾ dealt with the selection of favorable organic media for the reaction, and it was confirmed that the crude lipase preparation from *Ps. fragi* 22.39 B showed higher activity in hydrocarbons such as *n*-hexane, isooctane and cyclohexane, among the many organic solvents examined. In the present paper, we report the substrate specificity and influence of repeated use of the enzyme for ester synthesis in *n*-hexane.

The crude lipase preparation (dry powder) used in this study was prepared as described in our previous paper.³⁾ The preparation contained only one kind of lipase and no other enzymes which had esterifying activity. This was confirmed by investigating the activity of the remaining material after removal of the lipase from the above enzyme preparation. The lyophilic activity of this lipase preparation was 202 units/mg of powder, as assayed at 37°C and pH 7.0 according to the method of Yamada.⁷⁾ One unit of the enzyme was defined as the amount which liberated 1 μ mol equivalent of fatty acid from olive oil per minute. The water content of this preparation was 5.4% (w/w), as measured by the Karl-Fischer method.⁸⁾

The reaction was performed as follows; into a 150 ml Erlenmeyer flask with a screw cap, 20 ml of *n*-hexane containing 2 mmol of alcohol and 2 mmol of carboxylic

acid was poured, and then 10 mg of the lipase was suspended in the mixture, followed by incubation at 25°C with constant shaking (100 oscillations/min). The reaction was stopped by diluting the mixture with 80 ml of ethanol, and then free carboxylic acid was immediately titrated with 0.5 N NaOH in ethanol. The degree of ester formation was calculated from the amount of acid consumed in the reaction mixture. The water content of *n*-hexane used as the reaction medium was 0.002% (v/v), as measured by the Karl-Fischer method. The synthesized esters were identified by thin-layer chromatography (TLC) and infrared spectroscopy. TLC was conducted on a silica gel plate (Type 60, Merck Co., Ltd.) with a solvent system of petroleum ether-diethyl ether-acetic acid (70:30:1, v/v). In order to investigate the influence of repeated use of the lipase, after performing the reaction in *n*-hexane with palmitic acid and cetyl alcohol as substrates, the used enzyme powder was recovered from the reaction mixture by centrifugation, and then washed with cold acetone and dried at room temperature for the next use. Through this operation, the enzyme could be recovered almost quantitatively. The supernatant which contained the reaction product was titrated in order to estimate the degree of ester formation.

Carboxylic acids and alcohols used as substrates were purchased from Tokyo Kasei Kogyo Co., Ltd. *n*-Hexane used as the reaction medium was obtained from Wako Pure Chemicals Co., Ltd.

At first, we examined the ester formation from various acids and alcohols by the lipase in *n*-hexane. When palmitic acid was used as the acyl donor, primary alcohols such as 1-butanol, benzyl alcohol and geraniol were esterified by the lipase faster than secondary alcohols such as 2-butanol and cyclohexanol. Within 4-hr incubation, the reaction with these primary alcohols had reached an equilibrium, showing an about 95% ester formation yield, while that with secondary alcohols had not. Tertiary alcohols such as tertiary butanol and terpineol were not esterified at all by the enzyme. On the other hand, although the lipase could catalyze ester synthesis from cetyl alcohol and fatty acids with a straight carbon chain, it did not esterify carboxylic acids having a branched carbon chain such as isovaleric acid and 2-hexyldecanoic acid nor ones having a benzene ring such as benzoic acid. After 4-hr incubation, the reaction between cetyl alcohol and medium or long chain fatty acids such as lauric acid and linoleic acid had reached an equilibrium, showing an about 94% ester formation yield, while that with short chain ones such as *n*-butyric acid did not. These results as to the substrate specificity of the lipase for ester synthesis in *n*-hexane were similar to those obtained with an aqueous solution in a previous study.²⁾ These facts indicate that the alcohol and acid specificities of the lipase as to esterification are not different between aqueous and organic media such as *n*-hexane.

Using various fatty acids and primary alcohols with saturated straight carbon chains as substrates for ester

FIG. 1. Relative Rates of Ester Synthesis from Various Fatty Acids and Primary Alcohols with a Saturated Straight Carbon Chain.

Lipase powder (10 mg) was added to 20 ml of *n*-hexane containing 2 mmol of fatty acid and 2 mmol of alcohol, and then the mixture was shaken at 25°C for 1 hr. The degree of ester formation was calculated from the amount of fatty acid consumed in the synthetic reaction. Relative activity was expressed in comparison with the yield of cetyl palmitate synthesis (61%), which was taken as 100%. A, reaction between palmitic acid and alcohols; B, reaction between cetyl alcohol and fatty acids.

TABLE I. INFLUENCE OF REPEATED USE OF THE LIPASE ON ESTER SYNTHESIS IN *n*-HEXANE

Lipase powder (10 mg) was added to 20 ml of *n*-hexane containing 2 mmol of palmitic acid and 2 mmol of cetyl alcohol, and then the mixture was shaken at 25°C. After 4-hr incubation, the lipase was recovered from the reaction mixture and dried according to the method described in the text, then the recovered enzyme powder was used for the next reaction. The yield of the synthesized ester was calculated from the amount of palmitic acid consumed in the reaction.

Number of uses	1st	2nd	3rd	4th	5th	6th
Yield (%)	96	91	92	90	88	86

inactivation of the lipase due to acidification of the aqueous reaction medium by the acids. On the other hand, such an influence of the acids seemed to be less in an organic solvent such as *n*-hexane, compared with in an aqueous solution. Therefore, the difference in lipase reactivity due to the carbon chain length of fatty acids was smaller in *n*-hexane compared with in an aqueous solution, and lower fatty acids such as acetic acid and *n*-butyric acid were esterified with alcohol.

The influence of the repeated use of the lipase on the ester formation from palmitic acid and cetyl alcohol in *n*-hexane was investigated using the enzyme preparation recovered from the reaction mixture. The results are summarized in Table I. Although the ester formation yields more or less decreased with the repeated use of the enzyme, presumably due to the slight loss of the lipase powder in the enzyme recovery, it was confirmed that this lipase was denatured by *n*-hexane and that the repeated use of the enzyme was practically possible in this reaction system.

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REFERENCES

- 1) T. Nishio, T. Chikano and M. Kamimura, *Agric. Biol. Chem.*, **51**, 181 (1987).
- 2) T. Nishio, T. Chikano and M. Kamimura, *Agric. Biol. Chem.*, **52**, 1203 (1988).
- 3) T. Nishio and M. Kamimura, *Agric. Biol. Chem.*, **52** in press (1988).
- 4) Y. Inada, H. Nishimura, K. Takahashi, T. Yoshimoto, A. R. Saha and Y. Saito, *Biochem. Biophys. Res. Commun.*, **122**, 845 (1984).
- 5) A. Zaks and A. L. Klivanov, *Proc. Natl. Acad. Sci.*

synthesis in *n*-hexane, the effect of the carbon chain length on the lipase activity were investigated. The relative rates of ester formation from palmitic acid and various primary alcohols (C₂–C₁₆) are shown in Fig. 1A. The reactivities of the lipase toward these alcohols with different carbon chain lengths were the same on the whole. Such a tendency was observed on reaction in an aqueous system using the purified lipase.²⁾ Figure 1B shows the relative rates of ester formation from cetyl alcohol and various fatty acids (C₂–C₁₆). The activity increased with increasing carbon number when short chain fatty acids (C₂–C₆) were used, but such a tendency was not observed with medium or long chain fatty acids (C₈–C₁₆). On reaction in an aqueous solution,²⁾ the activity of the lipase increased with increasing carbon number for fatty acids (C₂–C₁₆), but lower fatty acids such as acetic acid and *n*-butyric acid were scarcely esterified. This was thought to be caused by

U.S.A., **82**, 3192 (1985).

6) S. Osanai, *J. Jpn. Oil. Chem. Soc.*, **35**, 955 (1986).

7) K. Yamada, Y. Ota and H. Machida, *Nippon*

Nōgeikagaku Kaishi, **36**, 860 (1963).

8) J. Mitchell, Jr. and D. M. Smith, "Aquametry," 2nd Ed., Interscience Publishers Inc., New York, 1980.