Esterification Patterns of Lipases for Synthesizing Tricaproylglycerols in Organic Solvent

Dae Young Kwon*, Hyo Nam Song, and Suk Hoo Yoon

Food Chemistry and Physics Division, Korea Food Research Institute, Songnam, Kyongki, 463-050, Korea

ABSTRACT: To investigate the synthetic patterns of triglyceride (triacylglycerol) by lipases in organic solvent, esterification patterns of triglyceride, diglyceride, and monoglyceride were monitored at various reaction times with 10 lipases. As a model study, tricaprin was synthesized from glycerol and capric acids ($C_{10:0}$) in isooctane. Lipases that were known to give nonspecific hydrolysis in aqueous solvent, such as lipase from *Candida cylindracea*, Lipase OF-360 (from *C. rugosa*), and Lipase MY (*C. rugosa*) showed nonspecific synthesis of tricaprin in organic solvent (Group I). There are two groups for esterifying triglycerides in organic solvent with 1,3-specific lipases: one consists of the lipases from *Rhizomucor miehei*, *Pseudomonas aeruginosa* (Lipase PS), and *Chromobacterium viscosum* (Lipase CV) (Group II), and another (Group III) is represented by Lipase AP (*Aspergillus niger*), Lipase FAP-15 (*Rhizopus javanicus*), and Lipase D (*R. delemar*). Although both groups showed 1,3-specific hydrolysis in aqueous solvent, Group III has stricter 1,3-specificity for the synthesis of tricaprin from dicaprin.
JAOCS 74, 1287–1290 (1997).

KEY WORDS: Esterification pattern, lipase, lipase specificity.

In vitro synthetic reactions catalyzed by hydrolytic enzymes, such as protease, amylase and lipase, are almost impossible to make go in water (1), although these enzymes can catalyze hydrolysis and synthesis reversibly (2,3). The main reason why these enzymes can not catalyze the synthetic reaction in aqueous system is the unfavorable equilibria due to the high concentration of water (56 M) (1,4). Therefore, many investigators tried to find relevant reaction systems *in vitro* to perform synthetic reactions of ester compounds of fatty acids (5,6), carbohydrates (6), and peptides (7,8) or interesterification (9,10). The best system for such reactions is a nonaqueous organic solvent because of its advantage in removing the water (1,10).

For this reason, reaction characteristics or patterns of the enzymes in organic solvent are important factors to be considered during the synthesis of a biomolecule *in vitro*. However, reaction patterns and mechanisms of enzymes in organic solvents for synthesis or esterification of some molecules are not well known, while there are many papers on the reaction characteristics or performance of lipases in organic solvents (11–13). Especially for lipases, the esterification or synthetic specificities of triglyceride in organic solvent are not yet studied, although the hydrolytic specificity and its mechanisms in aqueous or emulsion systems have been well studied (14,15).

In our previous work (1,16), 10 lipases were selected from 21 lipases that showed good synthetic activities for glycerol and capric acid in organic solvents (1). They were classified into three groups according to the different synthetic patterns of tricaprin in isooctane (1). With three lipases from each respective group, interesterification patterns of tripalmitin with capric acid were investigated (16). The results showed that synthetic or interesterification patterns between the same 1,3-specific groups (Group II and Group III) in hydrolyzing the triglyceride (15–18) are not the same in organic solvent (1,16).

These two papers (1,16) gave us a hint that the synthetic specificity should be different than the hydrolytic specificity. Thus, to analyze the esterification patterns of lipases in terms of lipase specificity in an organic solvent, detailed time courses of esterification for tricaprin were estimated. The objective of this paper is to characterize the esterification patterns (synthetic patterns) of 10 lipases for producing mono-, di-, and tricaprin from capric acid and glycerol in organic solvent, and to compare the synthetic pattern to the hydrolytic pattern.

MATERIALS AND METHODS

Materials. All 10 lipases, Lipase CES (*Pseudomonas* sp.), Lipase AP (*Aspergillus niger*), Lipase PS (*P. aeruginosa*), Lipase L (*Candida lipolytica*), Lipase F-AP 15 (*Rhizopus javanicus*), Lipase D (*R. delemar*), lipase from *C. cylindracea*, lipase from *Rhizomucor miehei*, Lipase MY (*C. rugosa*), Lipase OF-360 (*C. rugosa*), and Lipase CV (*Chromobacterium viscosum*) were the same lipases as described in the previous paper (1). All chemicals for these experiments were also the same as described in our previous paper (1), unless otherwise specified.

Time courses of lipase-catalyzed esterification patterns. Time courses of lipase-catalyzed interesterification patterns of tricaprin, dicaprin, and monocaprin from capric acid and glycerol in organic solvent were constructed by determining each product at different reaction times. Enzymatic syntheses of tricaprin were performed according to the previous paper (1). The solvent used was isooctane, and the reaction temper-

*To whom correspondence should be addressed.
E-mail: DYKWN@chollian.Dacom.co.Kr.

ature was 25°C. After 0.5, 1, 2, 3, 5, 8, 12, and 24 h of reaction, aliquots were withdrawn, and the amounts of mono-, di, and tricaprin, and of capric acid were assayed by high-performance liquid chromatography (HPLC). Lipase activities were calculated by determining the amount of mono-, di-, and tricaprin produced. Analytical conditions of HPLC were the same as in the previous paper (1).

RESULTS AND DISCUSSION

The time courses of the synthetic pattern of each product at various reaction times for Groups I, II, and III lipases gave us detailed information regarding the profiles of synthesis of tricaprin, dicaprin, and monocaprin from capric acid in organic solvent.

Figure 1A shows the synthesis profile for *C. cylindracea* lipase (Group I) in isooctane. The Group I lipases, represented by lipases from *C. cylindracea* and *C. rugosa,* are known to be nonspecific in hydrolytic reactions (14,15). In the synthesis of tricaprin from glycerol and capric acid by *C. cylindracea* lipase, the first synthetic product (monocaprin) and the second product (dicaprin) were created rapidly in the first 2 h of reaction. Thereafter, monocaprin was converted readily to dicaprin, which did not easily react to the final product (tricaprin). Thus, after 5 h, dicaprin was accumulated substantially, while the amount of monocaprin decreased slightly, and only a small amount of tricaprin was produced (Fig. 1A).

Generally, lipases from *Pseudomonas* sp., *A. niger, P. aeruginosa, C. lipolytica, R. javanicus, Chr. viscosum, Rh. miehei,* and *R. delemar* are known to be 1,3-specific in the hydrolysis of triglycerides (14,15). It was believed that the reaction specificities for the synthesis of triglyceride in organic solvent by 1,3-hydrolytically specific lipases should not be different from those in aqueous solvent. Previous studies (1) had suggested that Lipase PS, Lipase CV, and lipase from *Rh. miehei* should be classified under Group II because they showed different profiles in synthesizing glycerides in organic solvent compared to the rest, which were classified under Group III. From our detailed time-dependent esterification patterns (Fig. 1B and Fig. 1C), we further confirmed that our temporal classification of the three groups of lipases (1,16) is correct. Group II lipases were good at tricaprin production, because dicaprin does not accumulate and readily goes to tricaprin (Fig. 1B). In the synthesis of triglyceride from glycerol and capric acid with *Rh. miehei*, all synthetic products were produced rapidly within the first 2 h, but thereafter, monocaprin and dicaprin were easily converted to the final product through additional binding of capric acid. Thus, after 4 h, tricaprin substantially accumulated while the amounts of dicaprin and monocaprin decreased.

Although the Group III lipases (Lipase AP, F-AP15, and D) have the same 1,3-positional hydrolytic specificities in an aqueous system as the Group II lipases (14), the synthetic pattern of tricaprin from capric acid by Group III lipases is obviously different from Group II lipases in organic solvent. Figure 1C shows that lipase from *A. niger* produced monocaprin rapidly within 4 h, which was also easily converted to dicaprin. However, the synthetic rate from monocaprin to dicaprin was more or less at par with monocaprin synthesis because both reached equilibrium after 10 h. Although they produced dicaprin maximally, other products, such as monocaprin and tricaprin, were also constantly produced throughout the reaction.

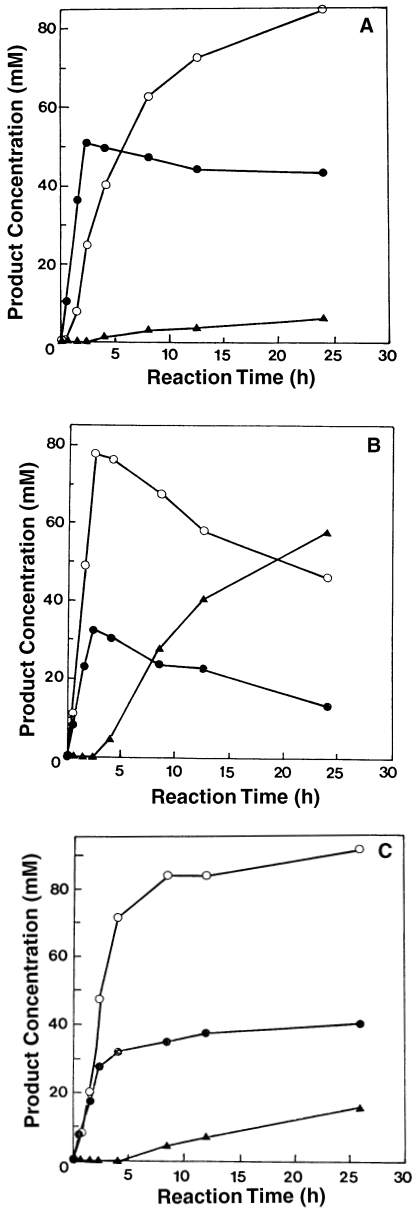

FIG. 1. Esterification pattern of medium-chain glycerides (tricaprin) in organic solvent by nonspecific Group I (A), Group II (B), and Group III lipases (C). Group I lipases were Lipase MY (*Candida rugosa*) and lipase from *C. cylindracea*), and Group II lipases were Lipase PS (*Pseudomonas aeruginosa*), Lipase CV (*C. viscosum*), and lipase from *Rhizomucor miehei.* Group III included Lipase AP (*Aspergillus niger*), Lipase FAP-15 (*Rhizopus javanicus*), and Lipase D (*R. delemar*). Symbols: ○, dicaprin; ●, monocaprin; ▲, tricaprin.

The present results and previous findings (1,16) showed that there were two different patterns of synthesis within the

same 1,3-hydrolytic specificities of lipases. To analyze the synthetic patterns of tricaprin for these lipases in an organic solvent and to compare them with their hydrolytic specificities in an aqueous system, synthetic routes of tricaprin for different specific lipases were studied. Reaction mechanisms, such as equilibria and kinetics of synthesis in organic solvent, may be different from the hydrolysis reaction of triglyceride in aqueous system.

Nonspecific lipases might take all routes in the synthesis of tricaprin from capric acid, just like hydrolysis does in aqueous system. Namely, nonspecific lipases synthesize first the α-monocaprin (*sn*-1- and *sn*-3-monocaprin) and *sn*-2-monocaprin from capric acid and glycerol at the same rate, and then they esterify not only α-monocaprin to α,β-dicaprin (*sn*-1,2- and *sn*-2,3-dicaprin) and *sn*-1,3-dicaprin but also *sn*-2-monocaprin to α,β-dicaprin. As a final step, they take both α,β-dicaprin and *sn*-1,3-dicaprin for esterification to tricaprin. In the synthesis of tricaprin by nonspecific lipase, we found that the synthesis of monocaprin and dicaprin from capric acid took place rapidly within 2 h. After 2 h, monocaprin production was negligible because of substrate limitation (glycerol, not capric acid) (1), and there was ready conversion of monocaprin to dicaprin. However, dicaprin did not go to the final product easily (Fig. 1A). In general, the reaction rates of final hydrolysis and synthesis for nonspecific lipase are assumed to be lower because the remaining reaction site in the substrate of the final reaction step is only one (one ester bond in monocaprin for hydrolysis; and one hydroxyl group of dicaprin for esterification) compared to the former steps (two sites in monocaprin and three sites in glycerol). All synthetic routes are open, though, to make tricaprin synthesis possible (14). However, the reason is not clear why the final reaction step in synthesis is actually slower than those of former steps.

If Group III and II lipases are the same 1,3-specific for synthesis in organic solvent, like hydrolysis in aqueous system, the synthetic pathway for 1,3-specific lipases is simple: The 1,3-specific lipase catalyzes only the esterification on the *sn*-1 or *sn*-3 position of glycerol. Thus, it catalyzes esterification of α,β-dicaprin but not *sn*-1,3-dicaprin, because it can not attack the *sn*-2 position of dicaprin. From capric acid, one isomer of monocaprin (α-monocaprin) is produced, while the other isomer (*sn*-2-monocaprin) is not easily produced. The 1,3-specific lipase can catalyze α-monocaprin and *sn*-2-monocaprin to *sn*-1,3-dicaprin and α,β-dicaprin, respectively. Such specificity produces only *sn*-1,3-dicaprin, which can not be used for further esterification to synthesize tricaprin. For further enzymatic reaction, *sn*-1,3-dicaprin must be nonenzymatically isomerized to α,β-dicaprin. (18). Only the resulting α,β-dicaprin can be esterified to tricaprin (14,15).

Group III lipases easily synthesized α-monocaprin because two reaction sites (*sn*-1 and *sn*-3) in glycerol are active to esterify with capric acid despite the fact that there is only one route to monocaprin. They synthesize easily *sn*-1,3-dicaprin from the large amount of α-monocaprin. Probably, the main reason for the slow reaction from dicaprin to tricaprin is that 1,3-specific lipase cannot esterify capric acid to the *sn*-2 position of *sn*-1,3-dicaprin, although *sn*-1,3-dicaprin was fruitfully produced. Thus, for further synthesis to tricaprin, *sn*-1,3-dicaprin must be isomerized to α,β-dicaprin nonenzymatically (18). This nonenzymatic isomerization must be the rate-limiting step of tricaprin synthesis. These results confirm that Group III lipases are so strict in the synthetic specificity for tricaprin as well as in hydrolysis of tricaprin or other triglycerides (3,14,16).

For 1,3-specific Group II lipases, results showed that the reaction rate for tricaprin synthesis should be at least the same or faster than for dicaprin and monocaprin syntheses, while the hydrolysis rate from monocaprin to capric acid was slow because isomerization is necessary for further hydrolysis. During synthesis, monocaprin has two reaction sites, whereas in hydrolysis, monocaprin has only one cleavage site in monocaprin. Thus, a high synthetic rate was observed. This result suggests that 1,3-specific Group II lipases should not be strictly specific in synthesizing tricaprin from dicaprin. Group II lipases must catalyze synthetic reactions by using both routes from *sn*-1,3-dicaprin and α,β-dicaprin before nonenzymatic isomerization from *sn*-1,3-dicaprin to α,β-dicaprin, as described above (4,18). In fact, without the lipase attacking the *sn*-2 hydroxyl group of *sn*-1,3-dicaprin for producing tricaprin, Group II lipase cannot reach over 80% conversion yield (>40% tricaprin) with only nonenzymatic isomerization (2,18). However, it is not clear whether *sn*-1,3-dicaprin is less specific than α,β-dicaprin for synthesizing tricaprin by lipase in organic solvent, and it is still unclear if only another α-position of α-monocaprin can be esterified by these lipases directly or if simultaneous isomerization of α-monocaprin to β-monocaprin takes place. Only chiral analyses between 1,3-dicaprin and α,β-dicaprin or between α-monocaprin and β-monocaprin during the synthetic reaction will solve these questions. At present, however, it is still beyond our accessibility to do this experiment.

In conclusion, it has been confirmed that lipases that have 1,3-specific hydrolytic activities may have different specificities in the synthesis of triglyceride in organic solvent.

REFERENCES

1. Kwon, D.Y., H.N. Song, and S.H. Yoon, Synthesis of Medium-Chain Glyceride by Lipases in Organic Solvent, *J. Am. Oil Chem. Soc. 73*:1521–1525 (1996).
2. Ergan, E., M. Trani, and G. Andre, Production of Glycerides from Glycerol and Fatty Acid by Immobilized Lipases in Nonaqueous Media, *Biotechnol. Bioeng. 35*:195–200 (1990).
3. Voet, D., and J.G. Voet, in *Biochemistry*, John Wiley & Sons, New York, 1990, pp. 393–768.
4. Goderis, H.L., G. Ampe, M.P. Feyten, B.L. Fouwe, W.M. Guffens, S.M.-Van Cauwenbergh, and P.P. Tobback, Lipase-Catalyzed Ester Exchange Reactions in Organic Media with Controlled Humidity, *Biotechnol. Bioeng. 30*:258–266 (1987).
5. Zaks, A., and A.M. Klibanov, Enzymatic Catalysis in Nonaqueous Solvents, *J. Biol. Chem. 263*:3194–3201 (1988).
6. Akoh, C.C., and B.G. Swanson, Synthesis and Properties of Alkyl Glycoside and Stachyose Fatty Acid Polyester, *J. Am. Oil Chem. Soc. 66*:1295–1301 (1989).

7. Aso, K., Enzymatic Approach to the Synthesis of a Lysine-Containing Sweet Peptide, *N*-Acetyl-L-Phenylalanyl-L-Lysine, *Agric. Biol. Chem. 53*:729–733 (1989).
8. Margolin, A.L., D.F. Tai, and A.M. Klibanov, Incorporation of D-Amino Acids into Peptides *via* Enzymatic Condensation in Organic Solvents, *J. Am. Chem. Soc. 109*:7885–7887 (1987).
9. Foglia, T.A., K. Petruso, and S.H. Feairheller, Enzymatic Interesterification of Tallow–Sunflower Oil Mixtures, *J. Am. Oil Chem. Soc. 70*:281–285 (1993).
10. Zaks, A., and A.M. Klibanov, The Effect of Water on Enzyme Action in Organic Media, *J. Biol. Chem. 263*:8017–8021 (1988).
11. Klibanov, A.M., Enzyme Work in Organic Solvents, *Chemtech 16*:354–359 (1986).
12. Kwon, D.Y., Synthesis of Stereoisomer Trifluoroethylmandelates and Their Stereospecificity for the Uses as the Substrate of Lipases in Organic Solvent, *J. Korean Chem. Soc. 36*:125–130 (1992).
13. Russel, A., and A.M. Klibanov, Inhibitor-Induced Enzyme Activation in Organic Solvents, *J. Biol. Chem. 263*:11624–11626 (1988).
14. Macrae, A.R., Lipase-Catalyzed Interesterification of Oils and Fats, *J. Am. Oil Chem. Soc. 60*:291–294 (1983).
15. Macrae, A.R., Microbial Lipases as Catalysts for the Interesterification of Oils and Fats, in *Biotechnology for the Oils and Fats Industry*, edited by C. Ratledge, P. Dawson, and J. Rattray American Oil Chemists' Society, Champaign, 1984, pp. 189–198.
16. Kwon, D.Y., E.P. Casa, and L.J. Pham, Lipase-Catalyzed Interesterifcation of Tripalmitin and Capric Acid in Nonaqueous System, *Foods & Biotechnol. 5*:349–352 (1996).
17. Schuch, R., and K.D. Mukherjee, Interesterification of Lipids Using an Immobilized *sn*-1,3-specific Triacylglycerol Lipase, *J. Agric. Food Chem. 35*:1005–1008 (1987).
18. Bezonana, G., Some Properties of Extracellular Lipase from *Rhizopus arrhizus, Lipids 9*:166–172 (1974).

[Received November 1, 1996; accepted May 11, 1997]