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Synthesis of Either Mono- or Diacylglycerol from High-Oleic Sunflower Oil by Lipase-Catalyzed Glycerolysis

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The difference in reaction conditions between the syntheses of mono- and diacylglycerols (MAG and DAG) was elucidated in terms of the enzymatic glycerolysis of high-oleic sunflower oil containing 89% oleic acid. The most efficient lipases were *Pseudomonas* lipoprotein lipase and the lipase from *Pseudomonas cepacia* for the MAG and DAG syntheses, respectively. In each case, the glycerol amount to be added to maximize the yield was 1.5-fold larger than the stoichiometric amount that is necessary to complete the glycerolysis reaction. The addition of a small amount of acetone to the reaction mixture was only slightly effective on the yield of MAG. The control of the reaction temperature was very important, and the critical temperature, below which the yield of MAG or DAG is significantly increased, was found to be lower for DAG synthesis than for MAG synthesis. The reaction time that was required to obtain a maximum yield was about 80 h for MAG synthesis, while it was 6-fold longer for DAG synthesis. The content of MAG and monooleoylglycerol approached 90 and 80% in the lipid reaction products, respectively. On the other hand, the content of DAG was 82%, of which the fatty acid composition was similar to that of the original oil.

Keywords: acetone, monooleoylglycerol, glycerolysis, high-oleic sunflower oil, lipase, monoacylglycerol, diacylglycerol

A large quantity of monoacylglycerols (MAG) is used as emulsifying agents in the manufacture of foods and cosmetics. MAG is usually made by the glycerolysis reaction of edible fats and oils at a very high temperature above 200°C, which is catalyzed with inorganic alkali such as sodium methoxide. To prevent the generation of dark-colored by-products with undesirable flavors, low-temperature MAG synthesis using lipase has been attempted by many researchers. There are two main enzymatic pathways to yield MAG; ester synthesis from free fatty acid and glycerol (Tsujisaka *et al.*, 1977; Hoq *et al.*, 1984; Schuch & Mukherjee, 1989; Akoh *et al.*, 1992; van der Padt *et al.*, 1992; Berger & Schneider, 1992b; Li & Ward, 1993), and glycerolysis of edible fats and oils (Yamane *et al.*, 1986a, b; Holmberg *et al.*, 1989; Yang *et al.*, 1994a, b). McNeill *et al.* (1990, 1991a, b) reported that a high yield of MAG (70-90%, depending on the kind of material) could be obtained by the enzymatic glycerolysis, if the reaction vessels were stepwise cooled, for example, from 50 to 40°C with beef tallow.

Diacylglycerols (DAG) are also synthesized by chemical glycerolysis of edible fats and oils, and used in the processes for foods, cosmetics, etc. However, only a few papers have been published that specifically attempted a high yield of DAG using enzymatic methods (Berger *et al.*, 1992a). The difference in the reaction conditions between MAG and DAG syntheses from high-oleic sunflower oil by lipase-catalyzed glycerolysis is described in this work.

Abbreviations: DAG, diacylglycerol; LPL, lipoprotein lipase; LPS, Lipase PS; MAG, monoacylglycerol; TAG, triacylglycerol.

Materials and Methods

Measurement of lipase activity Lipase activity was measured at 37°C using the method described by Ota and Yamada (1966), with an emulsion emulsified with polyvinyl-alcohol. One unit of lipase was defined as the amount of the enzyme that released 1 μmole of fatty acid from olive oil per min.

Enzymatic glycerolysis reaction A small amount (50 or 100 μl) of deionized water was dissolved in glycerol, and lipase powder (500 units per 1 g of oil) was suspended in the solution. After adding 6.87 g of high-oleic sunflower oil, the mixture was agitated by a magnetic stirring at 500 rpm at indicated temperatures in a 50 ml flat-bottomed glass vessel (35 mm i.d.×60 mm high) stoppered tightly with a screw cap, till it became solid, and then maintained without stirring.

Analysis of reaction products During the course of the reaction, samples of approximately 100 mg were intermittently transferred from the reaction vessel to a 10 ml screw-capped centrifuge tube. Three milliliter of chloroform were poured into the tube, and it was vigorously shaken for 2 min and left in a freezer for about 1 h. After adding 0.5 ml of deionized water, the mixture was shaken for 2 min and centrifuged at 2,400 rpm for 5 min. The lower chloroform layer was taken out and rinsed with 0.5 ml of deionized water. The combined water layer was extracted twice with 3 ml of chloroform each. The resultant chloroform layers were collected and concentrated with a rotary evaporator, and the concentrate was dehydrated with anhydrous sodium sulfate.

Analysis of the lipid product was done using a TLC/FID

autoanalyzer (Iatroscan TH-10, Iatron Laboratories, Tokyo). Chromarod S-III quartz rods were dipped in 3% boric acid solution and dried. One microliter of the lipid product solution was put on them and developed in a solvent mixture of benzene, chloroform and acetic acid (70 : 30 : 2 by volume). The separated bands of tri-, di- and monoacylglycerols, and free fatty acid were scanned by the autoanalyzer, and the results are expressed as % peak areas.

The fatty acid composition of the synthesized mono- and diacylglycerols, which was purified on a silica gel plate by thin layer chromatography, was measured by gas-liquid chromatography. The methyl esters of the fatty acid were prepared by the method using boron trifluoride/methanol.

Reagents Refined high-oleic sunflower oil was a gift from Amagasaki Works, NOF Corporation, Osaka. Its characteristics were as follows: acid value, 0.15; saponification value, 190.8; and iodine value, 80.8. The fatty acid composition was 2.9% palmitic, 2.8% stearic, 89.4% oleic and 3.4% linoleic acids. Lipase PS (LPS) from *Pseudomonas cepacia* was a gift from Amano Pharmaceutical Co., Ltd., Aichi (optimum pH 6.0 and specific activity 27 units/mg), and Lipoprotein Lipase (LPL) from *Pseudomonas* sp. was purchased from Toyobo Co., Ltd., Osaka (optimum pH 8.0 and specific activity around 1200 units/mg). Glycerol was purchased from Nacalai Tesque, Inc., Kyoto. All organic solvents used were dehydrated overnight with Molecular Sieves 3A 1/16 (Wako Pure Chemical Industries, Ltd., Osaka).

Results and Discussion

Selection of lipase for glycerolysis Two kinds of bacterial lipases from pseudomonad were selected and compared to each other in the two cases with the purpose that glycerolysis was to specifically synthesize MAG or DAG (Fig. 1).

Sunflower oil and glycerol, which are immiscible with each other, were made into an emulsion by magnetic stirring at 35°C. In the case of Fig. 1A, the MAG content in lipid products was not increased beyond 40%, if the reaction temperature was not lowered after a certain time during glycerolysis, as reported by McNeill *et al.* (1990, 1991a, b). The emulsion became solidified when the temperature was lowered. However, the MAG content was gradually increased in a solidified state without stirring, and reached about 80%. Lipoprotein lipase was fairly better than Lipase PS for the synthesis of MAG. The activity of LPS seems to be more inhibited by glycerol than that of LPL.

The synthesis of DAG is shown in Fig. 1B. The addition of glycerol and deionized water to the reaction mixture were reduced, compared with the case of MAG, and the temperature was stepwise controlled. The DAG content decreased through the temperatures of 15 and 10°C, but it was efficiently increased by further cooling to 5°C (about 80%). LPS was faster than LPL during the DAG synthesis, when the temperature was maintained at 5°C.

Acylglycerol composition during enzymatic glycerolysis As shown in Fig. 2A, MAG synthesis was done using LPL through a program of temperature control. The free fatty acid peak produced was rarely detectable with the TLC/FID

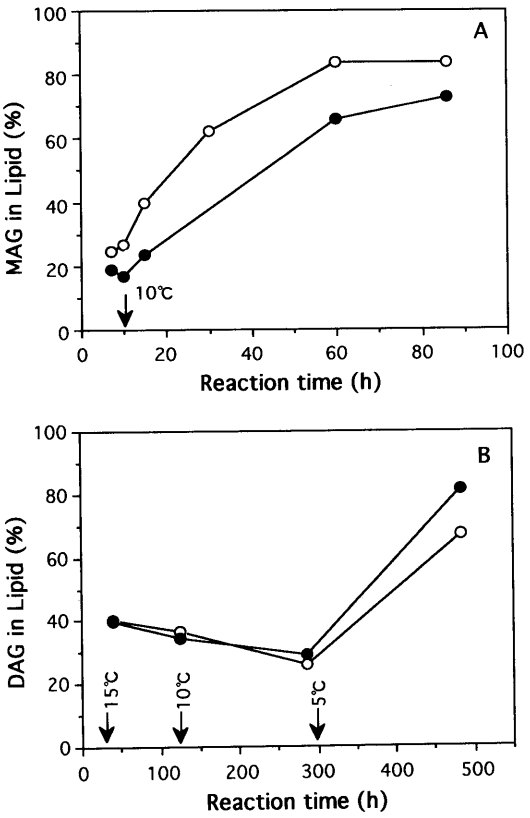

Fig. 1. Increase in mono- or diacylglycerols during glycerolysis of high-oleic sunflower oil catalyzed with two kinds of pseudomonas lipases. (A) Synthesis of monoacylglycerol. The reaction mixture contained 6.87 g of sunflower oil, 1.47 g of glycerol, 100 μl of deionized water and 3,400 units of lipase. Incubation temperature was 35°C up to 10 h and then lowered to 10°C. (B) Synthesis of diacylglycerol. The reaction mixture contained 6.87 g of sunflower oil, 0.368 g of glycerol, 50 μl of deionized water and 3,400 units of lipase. Incubation temperature was 35°C for 0–25 h, 15°C for 25–125 h, 10°C for 125–300 h, and finally 5°C for more than 300 h. ○ lipoprotein lipase, ● lipase PS.

autoanalyzer; so, it was ignored in the figures to simplify the progress of the reaction. MAG was hardly increased by the treatment at 15°C, but it was quickly synthesized at 10°C up to nearly 90%. Therefore, the critical temperature, Tc, designated by McNeill *et al.* (1990, 1991a), was likely between 10 and 15°C for the glycerolysis of high-oleic sunflower oil.

The results of the DAG synthesis are given in Fig. 2B using LPS. MAG was slightly increased at 10°C at the cost of DAG, which is contrary the purpose of this experiment. However, DAG was efficiently increased up to 80% at the lower temperature of 5°C, both TAG and MAG were decreasing. It is suggested that Tc is a little lower in the DAG synthesis than in the MAG synthesis.

Mono- and dioleoyl glycerols would be present as main reactants in the reaction mixtures. Physical properties such as melting point of the reactants is considered to be related to the values of T_c. The melting points of the mono- and dioleoyl glycerols are approximately 36 and 22°C, respectively. It can, therefore, be presumed that a lower temperature is required to obtain a high yield of DAG.

Effect of the molar ratio of glycerol to oil on the yield of MAG and DAG Stoichiometry predicts that 2 moles

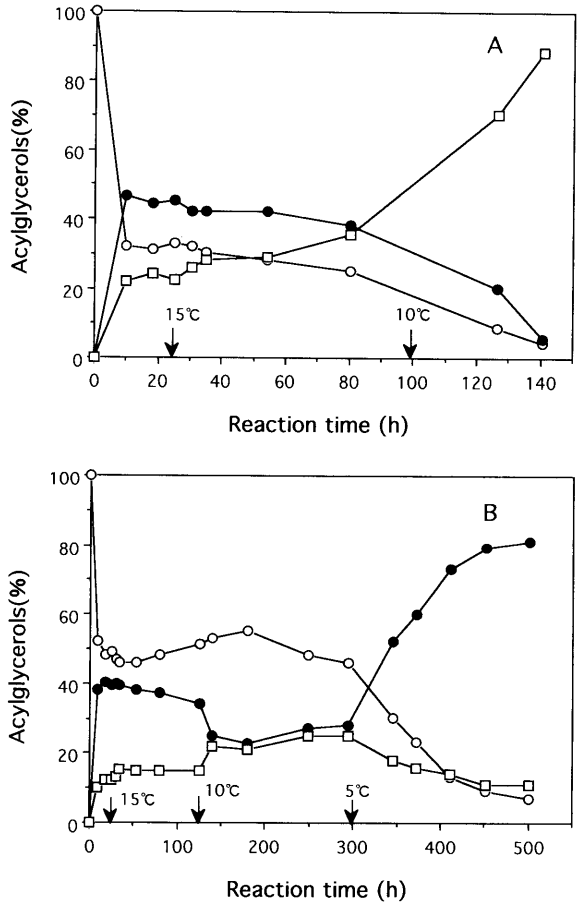

Fig. 2. Acylglycerol composition during enzymatic glycerolysis. (A) Synthesis of MAG using LPL. The reaction mixtures were the same as in Fig. 1A, and were incubated at 35, 15 and 10°C during the time, 0–25, 25–100 and 100–150 h, respectively. (B) Synthesis of DAG using LPS. The reaction mixtures were the same as in Fig. 1B, and were similarly incubated. □ MAG, ● DAG, ○ TAG.

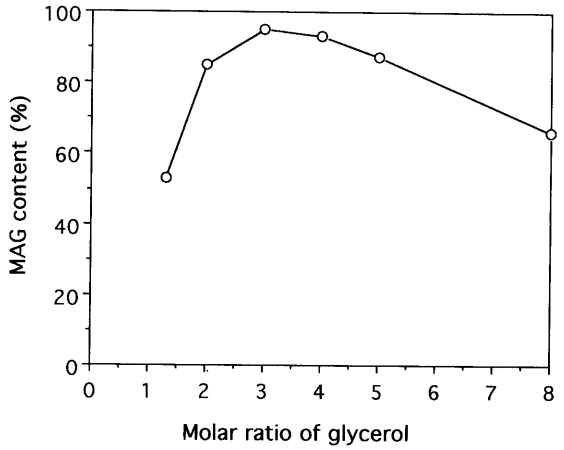

Fig. 3. Yield of monoacylglycerol with varying molar ratio of glycerol to sunflower oil. The reaction mixture was composed of 6.87 g (8 mmol) of sunflower oil, indicated amounts of glycerol (0.74 g corresponds to 8 mmol), 100 μl of distilled water and 2.9 mg (ca. 3,400 units) of LPL. The temperature programming was as 35°C (0–7 h), 15°C (7–18 h) and 10°C (more than 18 h). Each MAG content in total acylglycerol was measured after 80 h of glycerolysis.

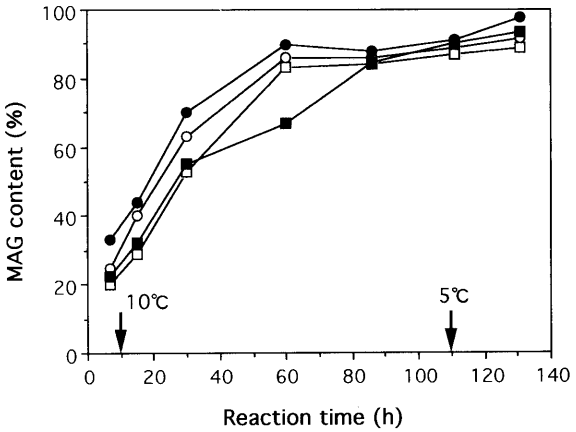

Fig. 4. Effect of organic solvents on the MAG synthesis with LPL. The reaction mixture was composed of 6.87 of sunflower oil, 2.21 g of glycerol. One-hundred microliters of distilled water, 3 mg of LPL and 1 ml of an organic solvent. The temperature programming was as 35°C (0–10 h), 10°C (10–110 h) and 5°C (110–130 h). ○ without organic solvent, ● acetone, ■ isooctane, □ cyclohexane.

and 0.5 mole of glycerol is necessary to complete the synthesis of MAG and DAG from 1 mole of TAG, respectively. Figure 3 shows the effect of the amounts of glycerol added to sunflower oil to synthesize MAG. As a result, excess glycerol was required, and a maximum yield of MAG (ca. 95%) was obtained with a 1.5-fold amount of glycerol. Similarly, 0.75 mole of glycerol was best for 1 mole of the oil during the DAG synthesis.

The first reaction step in the glycerolysis catalyzed with lipase is assumed to be the formation of an acyl-enzyme intermediate, leaving an alcohol compound such as MAG and DAG from DAG and TAG, respectively. The acyl-enzyme will then be converted to free enzyme with glycerol as an acyl-acceptor, yielding a molecule of MAG. Such a role of glycerol during the glycerolysis must require its excess presence in the reaction medium.

Addition of organic solvent to lipase substrates We had expected a favorable shift in equilibrium towards MAG synthesis by mixing a small amount of organic solvent with the lipase substrates. Figure 4 shows the results obtained with acetone, isooctane and cyclohexane to synthesize MAG with LPL. Only acetone, which is soluble in both the oily and glycerol-water phases, was always effective for the MAG synthesis, and the yield of MAG was slightly higher than that without acetone at a final temperature of 5°C. On the other hand, the other two nonpolar hydrocarbons were inhibitory, especially during the early stage of the reaction.

The yield from the DAG synthesis catalyzed with LPS was not increased at all with the addition of an organic solvent such as acetone, isopropyl alcohol, diethyl ether, benzene or *n*-hexane.

Adachi *et al.* (1993) reported that acetone specifically affected LPL, so that the contents of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in sardine oil was increased during the enzymatic acidolysis reaction with EPA- and DHA-enriched fatty acid. It is likely that there was also

a specific interaction between acetone and LPL during the glycerolysis reaction.

Fatty acid composition of synthesized MAG and DAG

With the best reaction conditions, MAG can be synthesized with a yield of more than 90% from high-oleic sunflower oil. The fatty acid composition of the MAG fraction isolated from the final reaction mixture was analyzed by gas-liquid chromatography. The contents of the unsaturated and saturated fatty acids were hardly changed with and without acetone, as compared with the original oil; so, about 89% of the MAG fraction and more than 80% of total acylglycerols was monooleoylglycerol. This result was also the case for the DAG synthesis. It is concluded that the selective distribution of saturated fatty acids into MAG or DAG did not occur in contrast to the results of McNeill *et al.* (1992).

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