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RESEARCH ARTICLE

Production of 1, 3-Dioleoyl-2-Palmitoyl Glycerol as a Human Milk Fat Substitute Using Enzymatic Interesterification of Natural Fats and Oils

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Abstract Human milk fat substitutes were synthesized using non-preprocessed natural fats and oils with lipase. Enzymatic interesterification of tripalmitin and oleic acid was carried out in isooctane. The reaction reached equilibrium after 12 h and the reaction conditions were partially optimized as a molar ratio of tripalmitin to oleic acid of 1:5, an initial water content of 0.01 g/L, and a water removal time of 1 h. After interesterification of tripalmitin and oleic acid, the reaction products contained 55.2% 1,3-dioleoyl-2-palmitoyl glycerol (OPO). The OPO yield was 25.2% when commercial palm oil and camellia oil were used as substrates, and the OPO concentration was increased to 53.3% after fractional crystallization of the reaction product.

Keywords: human milk fat substitute, lipase, interesterification, animal fat, plant oil

Introduction

Human milk fat has a unique molecular fatty acid distribution with over 70% of total palmitic acid positioned at the *sn*-2 position. Therefore, the major triacylglycerol is 1,3-dioleoyl-2-palmitoyl glycerol (OPO) (1). Industrial enzymatic methods have been used to produce a human milk fat substitute (HMFS) due to a stereospecific reaction ability (2,3). Tripalmitin and/or palm stearin have been used as a palmitic acid source, whereas free oleic acid has been used as an oleic acid source in the commercial HMFS manufacturing process (4-7). The concentrated reaction substrates (tripalmitin, palm stearin, and oleic acid) are beneficial for production of HMFS at higher reaction rates and production yields than for non-preprocessed natural palm oil and olive oil (8). Preparation of concentrated reaction substrates, however, increases the processing cost. Camellia oil and chufa oil (also called earth almond oil) are known to contain large amounts of oleic acid and can be used as oleic acid sources, in addition to olive oil (9,10). The objectives of this study were 1) to determine reaction yields of OPO when non-preprocessed natural fats and oils were used, and 2) to compare reaction yields using novel oleic acid sources of camellia oil and chufa oil rather than the conventional source of olive oil.

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Materials and Methods

Materials *Rhizomucor miehei* lipase (Lipozyme IM-20, >30 units/mg protein) was obtained from Novozymes Co., Ltd. (Bagrsbaerd, Denmark). The IM-20 lipid standards tripalmitin (PPP), *sn*-1,2-dipalmitoyl-3-oleoyl-rac-glycerol (PPO), *sn*-1,3-dipalmitoyl-oleoyl glycerol (POP), *sn*-1,2-dioleyl-palmitoyl glycerol (OOP), *sn*-1,3-dioleoyl-2-palmitoyl glycerol (OPO), oleic acid, triolein (OOO), and olive oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Authentic samples of fatty acid methyl esters were also purchased from Sigma. A molecular sieve (3Å, 1/16 inch) was purchased from Yakurt Pure Chemical Co. (Osaka, Japan). Silica Gel 60 (70-230 mesh ASTM) was purchased from Merck (Darmstadt, Germany). All solvents and reagents used were of HPLC or analytical grade unless otherwise specified. Palm oil was supplied by the Malaysian Palm Oil Board (Kuala Lumpur, Malaysia), and

lard was obtained from Lotte Samkang (Seoul, Korea). Camellia oil was purchased from a local market in Seoul, Korea and chufa oil was extracted using the conventional pressing method from earth almond seeds (9).

Analytical methods A gas chromatograph (Model 5890; Hewlett-Packard, Anaheim, CA, USA) was used to determine the fatty acid composition of camellia oil following AOCS method Ce 2-66 (11). A Supelcowax-10 capillary GC column (0.25 mm i.d.×60 m length, 0.2-μm film thickness; Supelco, St. Louis, MO, USA) was used for fatty acid analysis under conditions of an initial temperature of 175°C, a temperature increase rate of 3°C/min, a final temperature of 240°C, and a final hold time of 15 min. The temperatures of the injector and FID were 260°C and 270°C, respectively. The flow rate of the helium carrier gas was 25 mL/min, and samples were injected in split mode at a split ratio of 50:1.

Triacylglycerol molecular species were analyzed using an HPLC apparatus (Jasco, Tokyo, Japan) equipped with a model 980 HPLC pump, a 930 RI detector, and an 807-IT integrator using a Chrompack ChromSphere Lipids analytical silver-impregnated column (4.6 mm i.d.×250 mm length, stainless steel, 5 mm particle size; Varian, Bridgewater, NJ, USA) (12). The mobile phase was 0.5% acetonitrile in *n*-hexane with isocratic operation at a flow rate of 1.0 mL/min at 35°C. During the interesterification reaction, an aliquot was withdrawn from the reaction mixture using a microsyringe and injected into the HPLC for triacylglycerol analysis. Peaks in GC and HPLC chromatograms were identified based on comparison of retention times with authentic samples. The water content in the reaction mixture was determined using the Karl Fischer method (13).

Enzymatic interesterification Enzymatic interesterification reactions were carried out using 0.3 g of tripalmitin (0.372 mM) and 0.3 g of oleic acid (1.062 mM) in 5 mL of isooctane (14). Fifty mg of lipase (Lipozyme IM-20; Novozymes Co., Ltd.) was added to the reaction mixture followed by vigorous stirring using a vortex mixer at 40°C in a water bath (14,15). Intrinsic water in the isooctane was removed using addition of a molecular sieve before use.

During lipase-catalyzed interesterification of tripalmitin and oleic acid as a model system, aliquots were withdrawn at reaction times between 0-24 h as desired. The water content was adjusted to 0-0.2 g/L based on addition of water to isooctane to investigate the effect of the initial water content on interesterification,. The effect of the molar ratio of tripalmitin to oleic acid on productivity and OPO selectivity was investigated at molar ratios of 1:1, 1:3, 1:5, and 1:7 (tripalmitin:oleic acid) during a 12 h reaction. Silica gel (approximately 0.3 g, Slica Gel 60; Merck) was added to the reaction mixture to remove water produced during the reaction.

HMFS was also produced using palm oil and lard as palmitic acid sources, and camellia oil and chufa (earth almond) oil were used as oleic acid sources. To increase the OPO content in the reaction mixture after interesterification, reaction products was stored at 22°C for 12 h to fractionally crystallize PPO, PPP, and other triacylglycerols. Crystals formed were separated from the product mixture using filtration through Whatman No. 1 filter paper (Whatman International Co., Ltd., Kent, UK).

Statistical analysis All experimental values were reported as mean values of triplicate measurements. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) to ascertain significant differences between treatments. Significance was defined as $p<0.05$. Error bars and/or standard deviation were not shown when the standard deviation was less than 3%.

Results and Discussion

Fatty acid composition of fats and oils The fatty acid compositions of olive oil, camellia oil, chufa oil, palm oil, and lard are shown in Table 1. The fatty acid compositions of the fats and oils tested were all consistent with reported values (10,16). The oleic acid contents in camellia oil and chufa oil were 81.6 (by chromatogram area) and 69.7%, respectively. The oleic acid content in olive oil was 73.3%. The palmitic acid contents in palm oil and lard were 44.3 and 27.1%, respectively.

Partial optimization of interesterification conditions To determine the proper amount of oleic acid addition to tripalmitin, different molar ratios from 1:1 to 1:5 of tripalmitin to oleic acid were tested. With an increase in the molar ratio (tripalmitin:oleic acid from 1:1 to 1:5), OPO production increased from 11.2 to 38.2% (Fig. 1). No further increase of OPO production was observed at a molar ratio of 1:5. Therefore, the optimum molar ratio was 1:5. Production of PPO and OPP increased from 47.2 to

Table 1. Fatty acid compositions of fats and oils (unit: % wt)

	Olive oil	Camellia oil	Chufa oil	Palm oil	Lard
Palmitic acid	12.1	8.5	14.6	44.3	27.1
Stearic acid	2.8	2.0	3.5	4.6	11.0
Oleic acid	73.3	81.6	69.7	38.7	44.4
Linoleic acid	9.3	7.4	10.6	10.5	11.4
Linolenic acid	0.7	0.3	1.5	0.3	1.5
Arachidic acid	3.3	0.8	-	0.3	1.0

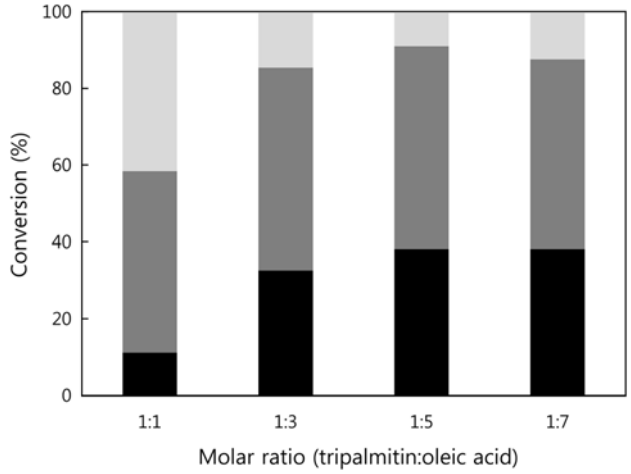

Fig. 1. Effect of the molar ratio of tripalmitin to oleic acid on triacylglycerol production for enzymatic interesterification in isooctane. ■, OPO; ■, PPO+OPP; ■, others

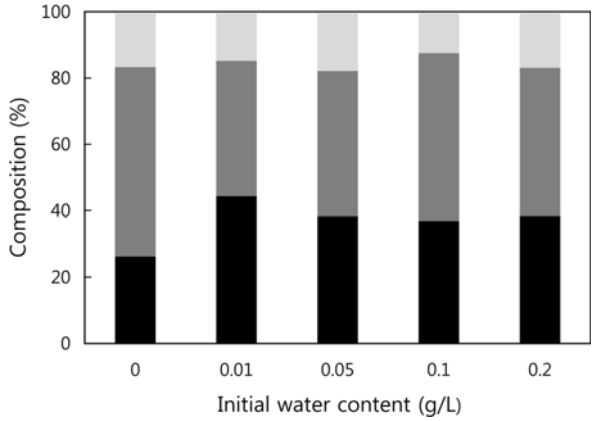

Fig. 2. Effect of the initial water content on triacylglycerol production for enzymatic interesterification in isooctane. ■, OPO; ■, PPO+OPP; ■, others

52.8% up to a ratio of 1:5, then decreased to 49.4% at a ratio value of 1:7. The dominant triacylglycerols produced during interesterification of tripalmitin and oleic acid at all molar ratio values tested were OPO and PPO+OPP.

The composition of the reaction mixture that produced OPO and other triacylglycerols was affected by the initial water content in the reaction system (Fig. 2). The maximum production of OPO was achieved at an initial water content of 0.01 g/L. The total integrated areas of each peaks obtained from HPLC analyses were 152, 183, 173, 176, and 154×10^6 with initial water contents of 0, 0.01, 0.05, 0.1, and 0.2 g/L. The hydrolytic activity of lipase is generally low at a low reaction mixture water content because water is necessary for attacking the ester bond in the hydrolysis reaction (17,18). The esterification reaction activity is, however, low with a high water content because water facilitates the hydrolysis reaction rather than the synthesis reaction. In other words, water is necessary for hydrolysis during the initial stage of lipase interesterification, but is not necessary for the actual esterification reaction that follows. Furthermore, water produced during the esterification reaction should be removed from the reaction mixture since water causes hydrolysis of triacylglycerol to diacylglycerol (19). Surplus water also causes agglomeration and inactivation of enzymes (20). It is, therefore, important to remove water during the late stage of interesterification.

When silica gel was added to the reaction mixture after a 1 h reaction time, a maximum production of OPO (53% conversion) was achieved (Fig. 3). When silica gel was added after 2 h of reaction, the production rate of OPO decreased. A similar pattern was observed with addition after 3 h. Production of PPO and OPP was decreased with addition of silica gel after 2 h of reaction than after 1 h (Fig. 3). The optimum time of silica gel addition, therefore, was after 1 h.

The optimum conditions for interesterification of tripalmitin and oleic acid were a molar ratio of tripalmitin to oleic acid of 1:5, an initial water content of 0.01 g/L, and a water removal time of 1 h. Under optimized conditions, OPO production with respect to reaction time is shown in Fig. 4. The tripalmitin content decreased sharply in the first 2 h, then the decrease rate was reduced up to 5 h, followed by a mostly constant rate thereafter. Production of PPO and OPP occurred quickly within 1 h, and increased slowly up to 3 h, remaining almost constant thereafter. OPO was predominantly produced during the first 5 h of reaction with a low increasing rate up to 55.2%. The reaction reached equilibrium after 12 h.

Production of HMFS using natural fats and oils The optimum molar ratio of tripalmitin to oleic acid (1:5) was used to calculate the substrate amounts of natural fats and oils. For interesterification of palm oil and camellia oil using triacylglycerol, which contains palmitic acid at the *sn*-2 position in palm oil, rates of 11 to 11.9% were achieved (21,22). The oleic acid content in camellia oil was 81.6% (Table 1). In this study, the *sn*-2 palmitic acid content was assumed to be 11.5%. To produce 1 mol of OPO, 1 mol of tripalmitin, and 2 mol of oleic acid were theoretically required. The optimum molar ratio of tripalmitin to oleic acid was, however, 1:5, which was higher than the theoretical value of 1:2, probably due to practical limitations of substrate migration to reaction sites.

The molecular weight (Mw) values of olive oil, camellia oil, and earth almond oil were calculated based on fatty acid compositions, and the Mw values of individual fatty acids. Palm oil and lard were used as sources of *sn*-2 palmitic acid oils. The *sn*-2 palmitic acid content in lard was reported to be 15.7%, comprising 85.3% of the total palmitic acid in lard (23). OPO production yields using

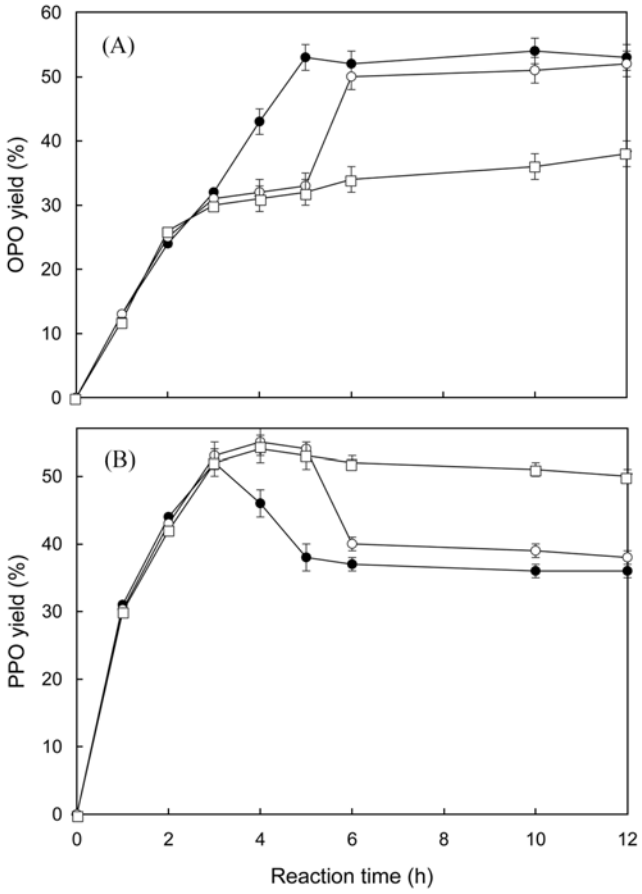

Fig. 3. Effect of the water removal time on triacylglycerol production for enzymatic interesterification in isooctane. □, no silica; ●, 1 h; ○, 3 h

Table 2. Effects of palmitic acid and oleic acid sources on production of OPO in enzymatic interesterification

Palmitic acid source	Oleic acid source	OPO concentration (%, area)
Tripalmitin	Oleic acid	55.2
Palm oil	Olive oil	21.8
	Camellia oil	25.2
	Earth almond oil	19.9
Lard	Olive oil	12.9
	Camellia oil	15.4
	Earth almond oil	9.0

different fats and oils are shown in Table 2. The highest production of OPO (55.2%) was achieved using tripalmitin and oleic acid as substrates. When palm oil and camellia oil were used, OPO production values were 25.2 and 21.8%. Using palm oil and olive oil, 21.8% production was achieved. When earth almond oil was used as an oleic acid source with palm oil, 19.9% OPO was produced. OPO production with lard as a palmitic acid source resulted in a lower production yield than with palm oil. Camellia oil used with lard resulted in 15.4% OPO production, followed

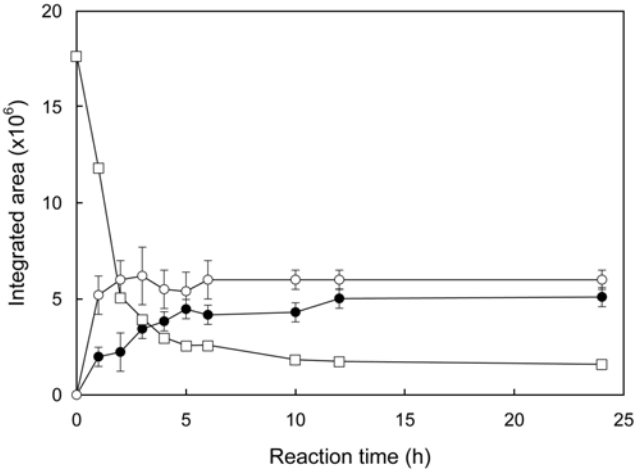

Fig. 4. Production of OPO and other triacylglycerols by enzymatic interesterification using tripalmitin and oleic acid in isooctane. ●, OPO; ○, PPO+OPP; □, PPP

by 12.9% with olive oil, and 9.0% with earth almond oil. Camellia oil was the most efficient oleic acid source for OPO production with both palm oil and lard. The oleic acid content at the *sn*-1 and 3 positions in camellia oil was 80.4% (10). Values for olive oil and chufa oil were 72 and 60.8%, respectively (9,24). The lipase used was 1,3-specific. Lipase can react more freely with oleic acid at the *sn*-1 and 3 positions than at the *sn*-2 position (15).

Palmitic acid contents at the *sn*-2 position in palm oil and lard were 11 to 11.9% (21,22), and 63.5% (25), respectively. The reaction temperature used for analysis was 40°C, and the melting point of lard was 48°C (26). The low production yield using lard with a higher palmitic acid content at the *sn*-2 position was related to a relatively low solubility in isooctane at the reaction temperature at 40°C.

The OPO content in commercial HMFS products, such as *Betapol*, is 53.5-73.9% (7). *Betapol* is produced using palm stearin and oleic acid sources. Palm stearin contains 50-60% tripalmitin (7) and has been used as a palmitic acid source for industrial production of *Betapol*. After the enzymatic interesterification and purification process, the final concentration of OPO in *Betapol* is obtained. The reaction mixture used in this study after interesterification still contained residual amounts of non-converted PPP, and the partially converted products PPO and OPP. After fractional crystallization of the reaction mixture of palm oil and camellia oil, the OPO content increased from 25.2% (by chromatogram area) to 53.3%. The production process of OPO as HMFS using natural palm oil and other oleic acid oils was possible as an alternative manufacturing process that required no pre-processing for preparation of special palm stearin, tripalmitin, and free oleic acid substrates, which increase production costs (27).

Disclosure The authors declare no conflict of interest.

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