Enzymatic Preparation and Oxidative Stability of Human Milk Fat Substitute Containing Polyunsaturated Fatty Acid Located at sn-2 Position

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Abstract: The development of human milk fat substitutes (HMFSs), rich in palmitic acid (16:0) at the sn-2 position of triacylglycerol (TAG) and rich in unsaturated fatty acids (FAs) (oleic acid, 18:1 and linoleic acid, 18:2) at the sn-1(3) positions, has gained popularity. In this study, HMFSs containing polyunsaturated fatty acids (PUFAs) predominantly at the sn-2 position were prepared, and their oxidation stabilities were compared. First, a non-PUFA-containing HMFS (NP-HMFS) was produced by enzymatic reactions using Novozyme® 435 and Lipozyme® RM-IM as the enzymes and lard as the raw material. Second, HMFSs, containing 10 % PUFA at the sn-2 or sn-1(3) position, were individually prepared by enzymatic reactions using lard and fish oil as raw materials. Here, sn-2-PUFA-monoacylglycerol (MAG) was extracted from the reaction solution using a mixture of hexane and ethanol/water (70:30, v/v) to produce high-purity sn-2-PUFA-MAG with 78.1 % yield. For the PUFA-containing HMFS substrates, comparable oxidation stability was confirmed by an auto-oxidation test. Finally, HMFSs containing 10 % or 2 % sn-1,3-18:1-sn-2-PUFA-TAG species were prepared by enzymatic reactions and subsequent physical blending. The oxidative stability of sn-1,3-18:1-sn-2-PUFA-HMFS was two-fold higher than that of 1/2/3-PUFA-HMFS in which each PUFA was located without stereospecific limitations in TAG. The removal of PUFA-TAG molecular species with higher concentrations of unsaturated units had a significant effect. In addition, the oxidation stability increased with the addition of tocopherol as an antioxidant. Thus, the combined use of two strategies, that is, the removal of PUFA-TAG molecular species with high concentrations of unsaturated units and the addition of antioxidants, would provide a PUFA-containing HMFS substrate with high oxidative stability.

Key words: human milk fat substitute, PUFA, enzymatic preparation, physical blend, oxidation stability

1 Introduction

Human milk fat (HMF) is a structured triacylglycerol (TAG) which has high amounts of palmitic acid (16:0), esterified at the TAG sn-2 position (ca. 40–60%) and, with preferential positioning of oleic acid (18:1) and linoleic acid (18:2) at the TAG sn-1,3-positions. In addition, HMF contains polyunsaturated fatty acids (PUFAs) such as arachidonic acid (20:4) and docosahexaenoic acid (22:6) at 0.5–1.0% and 0.1–1.0%, respectively, and they have a crucial role in the proper growth and development of infants. HMF with these characteristics is acknowledged as the ideal nutrition source for infants; however, in certain cases where mothers cannot or choose not to breastfeed, human milk fat substitute (HMFS) can be one of the choices of nutrition for the infants.

The development of HMFS with high amount of 16:0 at the sn-2 position and enriched with specific fatty acids such as PUFAs is of great interest as infant formulas as they mimic the physical, chemical, and nutritional properties of HMF. The preparations of PUFA-containing HMFSs with high 16:0 at the sn-2 position from raw materials via enzymatic esterification, such as interesterification and acidolysis reactions, under certain lipases have been widely studied. Special attention is being paid to HMFSs...
with high PUFA content at the sn-2 position, while PUFA distributions are almost equivalent at all the three positions of the TAG species in commercial HMFSs. Actually, PUFAs are mainly located at the sn-2 position of TAG species in HMFS, and the TAGs enriched with PUFA at the sn-2 position have better absorption, distribution, and metabolism in infants. A superior bioavailability of 22:6 at the sn-2 position compared with that at the sn-1 and sn-3 positions was observed in a tissue of hamster and in a rat model. Considering the high similarity to HMF, HMFSs containing PUFA at the sn-2 position are seemingly more valuable. To date, several researchers have developed some preparation protocols to obtain HMFS with high amount of PUFA at the sn-2 position. In order to prepare HMFS containing PUFA at the sn-2 position, Robles et al. reported a four-step process, which involves the use of acidolysis of tuna oil to synthesize structured TAG rich in 16:0 and 22:6 at the sn-2 position. In addition, Nagachinta and Akoh obtained HMFS with relatively high amount of PUFA at the sn-2 position by acidolysis of palm olein with a free fatty acid (FA) obtained from PUFA single-cell oils. More recently, Álvarez and Akoh obtained an HMFS, which contains capric acid mostly esterified at the sn-1,3 positions, and substantial amounts of 16:0, 22:0, and 20:4 at the sn-2 position, respectively.

Moreover, it has also been considered that the examination of the oxidation stability of HMFS is one of the important factors in milk manufacturing. In studies that have reported the oxidation stability of HMFS, typically, the removal of antioxidants from the natural sources during the modification protocol was often mentioned as a challenge. However, the dependence of oxidation stability on the molecular species of PUFA-containing TAG in the absence of antioxidants has not been fully investigated for HMFSs. Furthermore, it is well acknowledged that lipids react with molecular oxygen resulting in a free radical chain sequence, and the kinetics of lipid peroxidation, which is the primary reaction, highly depends on the type of fatty acids and their esters. Of the fatty acids and esters consisting of homoconjugated diene structure, including CH₂ centers that are flanked by two double bonds, the relative propagation rate constants are highly dependent on the number of the CH₂ center. Therefore, there are some challenges associated with PUFAs including the susceptibility to oxidation as a result of their high degree of unsaturation. In addition, the correlation between the molecular species of PUFA-containing TAGs and the oxidation stability has been well investigated for TAG substrates except HMFS. Some reports mentioned that the TAG species containing PUFA at the sn-2 position seem more stable, and some reports have made contrary assertions. These contradicting reports indicate that the oxidation stability can delicately depend on the composition including other low unsaturated fatty acids (LUFAs) and saturated FAs as well as the condition of the oxidation test. The different tendency can occur with oil products that have unique composition, such as HMFS. Therefore, depending on the molecular species, it is important to examine the oxidation stability of HMFSs containing PUFA at the sn-2 or sn-1(3) and the number of linking PUFA units in a TAG molecule.

In our previous report, an HMFS containing large amount of 16:0 at the sn-2 position and substantial amounts of 18:1 and 18:2 at the sn-1(3) position was prepared using lard substrate as the main source. In addition, an HMFS containing PUFA at the sn-1(3) position was prepared and the oxidation stability was compared with that of extracted oil from commercially available powdered milk for infants.

In the present study, we prepared HMFSs containing PUFA selectively at the sn-2 position and investigated the oxidation stability. Two HMFSs, sn-2-PUFA-HMFS and sn-1,3-18:1-sn-2-PUFA-HMFS, were prepared using two different protocols, in a three- or four-step reactions consisting of Novozyme® 435-mediated ethanolysis reaction, Lipozyme® RM-IM-mediated acidolysis reaction, and/or physical blending (Schemes 1a–c, 2a, and 2b). Furthermore, the oxidation stability of HMFSs containing PUFA was investigated by comparing the peroxide value (PV) with that of an HMFS which does not contain PUFA (NP-HMFS), an HMFS containing PUFA selectively at sn-1(3) position (sn-1(3)-PUFA-HMFS), and a PUFA-containing HMFS prepared by the physical blending of NP-HMFS and fish oil (1/2/3-PUSA-HMFS), in which a variety of PUFA-linking TAG species were included (Schemes 1e and 2c).

2 Experimental Procedures
2.1 Materials
2.1.1 Substrates.
In this study, the commercially available lard (containing vitamin E) (Megmilk Snow Brand Co., Ltd., Tokyo, Japan) was used as the main TAG substrate for HMFS. Reagents such as oleic acid (O: 18:1; 100%), linoleic acid (L: 18:2; 96.3%, 18:1; 3.7%), and linolenic acid (Ln: 18:3; 95.3%, 18:2; 1.1%, 18:1; 3.7%) were purchased from Tokyo Kasei Co., Ltd. and used as FA substrates. PUFA ethyl ester (PUFA-Et: DHA-Et, 22:6; 57.6%, 22:5; 26.8%, other FAs; 15.6%, Nisshin Pharma Inc., Saitama, Japan), which was purified by silica-gel column chromatography (hexane/diethyl ether = 85:15) to remove vitamin E, was used for the preparation of sn-1(3)-PUFA-HMFS. Fish oil rich in 22:6 (Fish oil: DHA46-RD, Ikeda Tohka Industries Co., Ltd., Hiroshima, Japan), which was purified by silica-gel column chromatography (hexane/diethyl ether = 85:15) to remove vitamin E, was used for the preparation of 2-PUFA-MAG. The lipid and FA compositions of the lard and PUFA-TAG substrates are shown in Table 1.
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Scheme 1 Preparations of intermediates and HMFS products via enzymatic reaction. (a) Preparation of sn-2-16:0-MAG via ethanolysis reaction using Novozyme® 435. (b) Preparation of NP-HMFS using Lipozyme® RM-IM. (c) Preparation of sn-2-PUFA-MAG via ethanolysis reaction using Novozyme® 435. (d) Preparation of sn-2-PUFA-HMFS using Lipozyme® RM-IM. (e) Preparations of sn-1(3)-PUFA-HMFS using Lipozyme® RM-IM.

Scheme 2 Preparations of intermediates and HMFS products via enzymatic reaction and/or physical blend. (a) Preparation of sn-1,3-18:1-sn-2-PUFA-TAG using Lipozyme® RM-IM. Preparations of (b-1) sn-1,3-18:1-sn-2-PUFA-HMFS containing 10% PUFA and (b-2) sn-1,3-18:1-sn-2-PUFA-HMFS containing 2% PUFA by physical blend. (c) Preparation of 1/2/3-PUFA-HMFS by physical blend.

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2.1.2 Enzymes
Novozyme® 435 (Candida antarctica, 10,000 PLU/g, regiospecificity: random, MIK Pharm Co., Ltd., Tokyo, Japan) was used for ethanolysis reaction. Lipozyme® RM-IM (Rhizomucor miehei, 150 IUN/g, regiospecificity at sn-1 and -3 position, Novozymes Japan Ltd., Chiba, Japan) was used for the esterification and interesterification reactions.

2.1.3 Reagents
All other reagents were commercially available special grades.

2.2 Preparation of HMFS
2.2.1 Enzymatic preparation of sn-2-16:0-MAG
sn-2-Palmitoyl(16:0)-MAG was prepared by Novozyme® 435-mediated ethanolysis using 1.0 g of lard in a method similar to that used in a previous study (Scheme 1a)[26]. The reactants containing lard (1.0 g), Novozyme® 435 (0.4 g), and ethanol (4.0 g) were stirred at 37°C with magnetic agitation at 550 rpm for 2.0, 2.5, 3.0, 3.5, or 4.0 h. As discussed in 3.1.1., 3.5 h was selected as the appropriate reaction time. At the end of the reaction, Novozyme® 435 was removed by filtration, and the solvent was evaporated under reduced pressure. At 3.5 h reaction time, after the addition of hexane (20 mL) at 60°C, the resultant precipitate at room temperature was filtered to produce sn-2-16:0-MAG (79.8% yield). The lipid and FA compositions of the sn-2-16:0-MAG are shown in Table 1.

2.2.2 Enzymatic preparation of NP-HMFS
An NP-HMFS, HMFS without PUFA, was prepared from lard substrate according to a previous report with a slight modification (Scheme 1b)[26]. To the mixture of lard (1.5 mmol) and sn-2-16:0-MAG (3.0 mmol), LUFA mixtures (6.0 mmol; O/L/Ln = 5:2:1, mol/mol/mol) and Lipozyme® RM-IM (30 wt% of total substrates) were added and dissolved completely in 10 mL of hexane. The enzymatic esterification and interesterification reactions were carried out at 37°C with agitation at 550 rpm for 0.75, 1.0, 1.25, or 2.0 h. A crude mixture obtained after filtration was concentrated by removing hexane. In this study, 1.0 h was the appropriate reaction time and the crude mixture obtained under 1 h reaction condition was purified by 7% water-containing Florisil® column chromatography (diethyl ether/hexane = 85/15, v/v) to give a TAG-containing fraction (NP-HMFS; 71.3% yield). Here, the condition of LUFA mixture (6.0 mmol, O/L/Ln = 5:2:1, mol/mol/mol) was determined by calculating the amounts of O, L, and Ln that were needed to meet the HMFS composition close to mother’s milk as described in our previous paper[26]. The lipid and FA compositions of the NP-HMFS are shown in Table 2.

2.2.3 Enzymatic preparation of sn-2-PUFA-MAG
sn-2-PUFA-MAG was prepared by Novozyme® 435-mediated ethanolysis (Scheme 1c), and two different protocols were individually carried out as subsequent purification methods. At first, the reactants containing PUFA-TAG (3.0

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**Table 1** Lipid and FA compositions in % of raw materials and sn-2-MAG products.

| Abbreviations | Lard | Fish-oil | sn-2-16:0-MAG | sn-2-PUFA-MAG | sn-2-PUFA-MAG |
|---------------|------|----------|---------------|---------------|---------------|
| Raw materials |      |          |               |               |               |
| FA type       | TAG  | DAG      | MAG           | FA            | Others        |
| 14:0          | 6.7  | 6.5      | 0             | 0             | 0             |
| 16:0          | 29.3 | 43.2     | 19.0          | 5.4           | 90.9          |
| 18:0          | 14.4 | 3.4      | 6.7           | 1.7           | 4.2           |
| 18:1          | 37.6 | 40.1     | 14.3          | 8.4           | 0             |
| 18:2          | 12.0 | 6.8      | 0             | 0             | 0             |
| 18:3          | 0    | 0        | 0             | 0             | 0             |
| 22:5          | 0    | 0        | 5.5           | 2.2           | 0             |
| 22:6          | 0    | 0        | 50.0          | 82.3          | 0             |
| Others        | 0    | 0        | 4.5           | 0             | 0             |

**Table 1** Lipid and FA compositions in % of raw materials and sn-2-MAG products.
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5 g, Novozyme® 435 (1.5 g), and ethanol (12.0 g) were stirred at 37°C with magnetic agitation at 550 rpm for 3.5 h. At the end of the reaction, Novozyme® 435 was removed by filtration, and the solvent was evaporated under reduced pressure. Next, the purification of sn-2-PUFA-MAG (1.0 g) was carried out with silica gel column chromatography (hexane/diethyl ether, 85:15, 100 mL and 100 ˚C ethanol 180 mL) or solvent extraction using 80, 70, 60, or 50 ˚C aqueous ethanol solution (5.0 g) three times. This produced 100% 2-PUFA-MAG in a yield of 72.8% for silica gel column chromatography and 92.4% sn-2-PUFA-MAG in a yield of 78.1% for the solvent extraction method (70 ˚C ethanol solution). The syntheses of HMFSs containing PUFA predominantly at the sn-2 position (2.2.4 and 2.2.5) were performed using the sn-2-PUFA-MAG substrate prepared with solvent extraction using 70 ˚C ethanol solution owing to the high yield and purity. The lipid and FA compositions of sn-2-PUFA-MAGs prepared by two different methods are summarized in Table 1.

2.2.4 Enzymatic preparation of sn-2-PUFA-HMFS

Lard (1.5 mmol), sn-2-16:0-MAG (3.0 mmol − X), LUFA mixtures (8.8 mmol; O/L/Ln = 5:2:1, mol/mol/mol), sn-2-PUFA-MAG (X = 0.15, 0.30, 0.45, 0.80 or 1.4 mmol), and Lipolyme® RM-IM (30 wt% of total substrates) were mixed in 10 mL of hexane and the mixture was agitated at 550 rpm for 1 h (Scheme 1d). At the end of the reaction, the filtration and evaporation of solvent under reduced pressure were performed to produce a crude reaction mixture. Next, the crude mixture was purified by 70 ˚C water-containing Florisil® column chromatography (diethyl ether/hexane 85/15, v/v) to give a TAG fraction. In case of the addition of 1.4 mmol sn-2-PUFA-MAG reactant, sn-2-PUFA-HMFS containing 10% PUFA was obtained with 68.3% yield. Here, the condition of LUFA mixtures (8.8 mmol, O/L/Ln = 5:2:1, mol/mol/mol) was determined by calculating the amounts of O, L, and Ln that were needed to produce HMFS composition close to that of mother’s milk. The lipid and FA compositions of the product are shown in Table 2.

An sn-1(3)-PUFA-HMFS was prepared from lard substrate according to the enzymatic preparation protocol of NP-HMFS described in 2.2.2 with a slight modification (Scheme 1e). Lard (1.5 mmol), sn-2-16:0-MAG (3.0 mmol), LUFA mixtures (4.6 mmol; O/L/Ln = 5:2:1, mol/mol/mol), PUFA-Et (0.15, 0.30, 0.45, 0.80 or 1.4 mmol), and Lipolyme® RM-IM (30 wt% of total substrates) were mixed in 10 mL of hexane and the mixture was agitated at 550 rpm for 1 h. After the filtration and evaporation of solvent under reduced pressure, the crude reaction mixture was

Table 2 Lipid and total FA compositions in % and the corresponding FA compositions at the sn-2 position, total PUFA in %, PUFA% at sn-2 position and average degree of unsaturation of HMFSs and PUFA-containing TAG substrates. These substrates were used for auto-oxidation test.

| Products | HMFS | HMFS | HMFS | TAG | HMFS | HMFS | HMFS |
|----------|------|------|------|-----|------|------|------|
| Abbreviations | NP-HMFS | sn-(3)-PUFA-HMFS | sn-2-PUFA-HMFS | sn-1,3-18:1-sn-2-PUFA-HMFS | sn-1,3-18:1-sn-2-PUFA-HMFS (10% PUFA) | sn-1,3-18:1-sn-2-PUFA-HMFS (2% PUFA) | 1/2-3-PUFA-HMFS (10% PUFA) |

Chemical structure:

| Substrate compositions | TAG | MAG | FA | Total | sn-2 | sn-2 | sn-2 | sn-2 | sn-2 | sn-2 | sn-2 | sn-2 | sn-2 |
|------------------------|-----|-----|----|-------|------|------|------|------|------|------|------|------|------|
| FA type                |     |     |    |       |      |      |      |      |      |      |      |      |      |
| 14:0                   |     |     |    |       |      |      |      |      | 4.6  | 5.2  | 4.4  | 3.5  | 4.1  | 1.4  |
| 16:0                   |     |     |    |       |      |      |      |      | 26.4 | 62.2 | 24.9 | 63.4 | 22.7 | 55.6 |
| 18:0                   |     |     |    |       |      |      |      |      | 12.1 | 5.3  | 8.5  | 4.6  | 6.4  | 2.1  |
| 18:1                   |     |     |    |       |      |      |      |      | 37.4 | 20.3 | 35.8 | 19.6 | 39.6 | 10.6 |
| 18:2                   |     |     |    |       |      |      |      |      | 17.2 | 6.9  | 15.8 | 8.9  | 15.7 | 2.3  |
| 18-3                   |     |     |    |       |      |      |      |      | 2.3  | 0.1  | 0.7  | 0.1  | 1.5  | 0.0  |
| 22:5                   |     |     |    |       |      |      |      |      | 0    | 0    | 2.6  | 0.0  | 0.2  | 0.0  |
| 22-6                   |     |     |    |       |      |      |      |      | 0    | 0    | 7.3  | 0.0  | 9.8  | 27.5 |
| PUFA% at sn-2          | 0.0  | 0.0  | 97.3 | 98.7  | 96.9 | 95.9 | 46.4 |
| Unsaturation degree    | 0.8  | 1.3  | 1.4  | 2.5   | 1.2  | 0.9  | 1.2  |

Table 2 Lipid and total FA compositions in % and the corresponding FA compositions at the sn-2 position, total PUFA in %, PUFA% at sn-2 position and average degree of unsaturation of HMFSs and PUFA-containing TAG substrates. These substrates were used for auto-oxidation test.

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purified by 7% water-containing Florisil® column chromatography (diethyl ether/hexane = 85/15, v/v) to give a TAG fraction. With the addition of 1.4 mmol PUFAEt reactant, HMFS containing 10% PUFA at sn-1 (3) position (sn-1 (3)-PUFA-HMFS) was obtained with 69.4% yield. The lipid and FA compositions of the sn-1 (3)-PUFA-HMFS are shown in Table 2.

2.2.6 Preparations of sn-1,3-18:1-sn-2-PUFA-HMFS via enzymatic synthesis and physical blending

At first, sn-2-PUFA-MAG (1.5 mmol), LUFAs substrates (O, 6.0 mmol), and Lipozyme® RM-IM (30 wt% of total substrates) were mixed in 10 mL of hexane, and the mixture was agitated at 550 rpm for 1 h (Scheme 2a). At the end of the reaction, Lipozyme® RM-IM was removed by filtration, and solvent was evaporated under reduced pressure. Next, the crude reaction mixture was purified by 7% water-containing Florisil® column chromatography (diethyl ether/hexane = 85/15, v/v) to give the sn-1,3-18:1-sn-2-PUFA-TAG in a yield of 86.3%. Arbitrary amounts of NP-HMFS was physically blended with sn-1,3-18:1-sn-2-PUFA-TAG completely, producing 10% sn-1,3-18:1-sn-2-PUFA-TAG or 2% sn-1,3-18:1-sn-2-PUFA-TAG-containing HMFS (Scheme 2b). The lipid and FA compositions of these products are shown in Table 2.

2.2.7 Preparation of 1/2/3-PUFA-HMFS using physical blend

Fish-oil (1.6 mmol) was physically blended with NP-HMFS (6.5 mmol) to produce PUFA-TAG containing HMFS (1/2/3-PUFA-HMFS) in which PUFAs are linked with TAG molecules by three units as a maximum (Scheme 2c). The lipid and FA compositions of these products are shown in Table 2.

2.3 Measurements

2.3.1 Estimation of lipid composition

The lipid composition of commercial materials and products was analyzed using an Iatroscan MK-6s thin-layer chromatography-flame ionization detector (TLC-FID) (LSI Medience Corporation, Tokyo, Japan). TLC-FID analysis was conducted using an Iatroscan MK-6s with a silica gel rod S-IV (LSI Medience Corporation, Tokyo, Japan) and a mixture of benzene/chloroform/acetic acid (35/15/1, v/v/v) as the development solvent.

2.3.2 Estimation of FA composition

FAs of acyl glycerol substrates were methyl-esterified as described by Jham et al. and subjected to gas chromatography (GC) analysis to determine its FA composition. GC analysis was performed using GC-2014 (Shimadzu, Kyoto, Japan) equipped with an FID detector and a fused silica capillary column (HR-SS-10, 0.25 mm × 25 m: Shinwa Chemical Industries, Ltd., Kyoto, Japan). The column temperature was programmed as follows; at first, the temperature was increased at 2°C/min from 170°C to 186°C, and then, the temperature was increased at 7°C/min from 186°C to 200°C. The temperatures of both the injector and the detector were set to 250°C for the analysis. 2.3.3 Estimation of FA composition at the sn-2 position of TAG

At first, the corresponding 2-MAG was prepared from the targeted TAG product by Novozyme® 435-mediated ethanolysis reaction. Briefly, TAG product (1.0 g) and Novozyme® 435 (2.5 g) were mixed with ethanol (3.5 g), and the reaction was carried out at 37°C by agitating at 550 rpm for 3 h. At the end of the reaction, the filtration and evaporation of solvent under reduced pressure were performed to produce a crude reaction mixture. Next, the crude mixture was purified by 7% water-containing Florisil® column chromatography (diethyl ether/hexane = 1/1, v/v) to give a corresponding sn-2-MAG. Subsequently, the FA composition of sn-2-MAG was measured as described in Section 2.3.2.

2.3.4 Evaluation of the oxidation stability

Oxidation stability at the primary step was evaluated by an auto-oxidation test. Briefly, each of the substrates was added to a petri dish (90 mm × 15 mm) and kept at 30 or 40°C in the dark. According to the standard method for the analysis of fats, oils, and related materials as prescribed by the Japan Oil Chemist’s Society® 26, 31, the PV (meq/kg) was evaluated in a time-course manner by a potentiometric titration method using a GT-200 General-Purpose Automated Potentiometric Titrator (Mitsubishi Chemical Analytech Co., Ltd, Kanagawa, Japan).

3 Results and Discussion

3.1 Enzymatic preparations of sn-2-PUFA-HMFS and sn-1 (3)-PUFA-HMFS and their oxidation stability

In this section, sn-2-PUFA-HMFS and sn-1 (3)-PUFA-HMFS were prepared via enzymatic synthesis and the oxidative stability was determined. At first, sn-2-16:0-MAG, NP-HMFS, and sn-2-PUFA-MAG were prepared. Here, sn-2-MAG substrates were prepared as the intermediate substances for the subsequent preparation of HMFS. It is well-known that Novozyme® 435 and Lipozyme® 435 (lipase B from Candida Antarctica, immobilized on a Lewatit VP OC 1600) are useful enzymes for the selective removal of FAs located at sn-1 (3) positions in a primary alcohol substituent, such as ethanol solvent, in a process known as ethanolysis® 26, 29, 32–34. In this study, Novozyme® 435 was used for ethanolysis reaction to prepare 2-MAG substrates from the corresponding TAG substrates. NP-HMFS, sn-2-PUFA-HMFS, and sn-1 (3)-PUFA-HMFS were prepared by Lipozyme® RM-IM-mediated reaction using lard and LUFAs in addition to sn-2-PUFA-MAG and/or sn-2-16:0-MAG. Subsequently, the oxidation stabilities of the HMFS substrates were compared.

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3.1.1 Enzymatic preparation of sn-2-16:0-MAG

For the preparation of sn-2-16:0-MAG-rich 2-MAG substrate from lard (Scheme 1a), the appropriate reaction time was determined. As shown in Fig. 1, the unreacted diacylglycerol (DAG) and/or TAG substrates remained in the shorter reaction time, whereas the amounts of MAG product started to decrease over 3–3.5 h. The occurrence of intra-exchange reaction from sn-2 of sn-2-MAG to sn-1 (3) and the subsequent hydrolysis reaction at sn-1 (3) was considered over 3–3.5 h. The highest percentage of 2-MAG composition was generated at 3.5 h, and hence, it was selected as the appropriate reaction time. The FA compositions of lard and sn-2-16:0-MAG are shown in Table 1. The lard contained over 40% 16:0 at the sn-2 position, and the FA composition of sn-2-16:0-MAG was over 90%, meaning that the purification protocol effectively removed other 2-MAG species.

3.1.2 Enzymatic preparation of NP-HMFS

NP-HMFS was prepared by the Lipozyme® RM-IM-mediated reaction using lard, 18:1, 18:2, 18:3, and sn-2-16:0-MAG rich 2-MAG substrate (Scheme 1b). Here, the optimum reaction time was determined. As shown in Fig. 2, the amount of MAG substrate increased in <1 h reaction time, whereas at >1 h reaction time, the amount reduced owing to the progression of hydrolysis, which must have come from the alternation of enzyme catalytic activity with changing water activity in the reaction system. Thus, the reaction temperature was determined as 1 h. As shown in Table 2, NP-HMFS substrates contained high composition of 16:0 located at the sn-2 and unsaturated FAs at the sn-1 and the sn-3 positions, which were comparable to that of HMFS which has high amounts of 16:0 esterified at the TAG sn-2 position (ca. 40–60%)\(^1\), with preferential positioning of 18:1 and 18:2 at the TAG sn-1,3-positions\(^1\)\(^2\). Therefore, the prepared HMFSs were enriched with 16:0 at sn-2 and unsaturated FAs at the sn-1 (3) positions.

3.1.3 Enzymatic preparation of sn-2-PUFA-MAG

The synthesis of sn-2-PUFA-MAG from fish oil was also carried out using the same reaction condition and time (3.5 h). Moreover, Zhang et al. recently reported the production protocol of sn-2-PUFA-MAG without column chromatography fractionation\(^2\). After enzymatic ethanolysis reaction, they obtained sn-2-22:6-MAG or sn-2-20:4-MAG with high purity from the reaction mixtures by solvent extraction from the hexane layer using mixed solvent of ethanol/water (85/15, v/v). Considering the previous reports, two purification protocols were compared in this study: typical column chromatography fractionation protocol and solvent extraction method without the column chromatography fractionation protocol. With regards to the solvent extraction method, the yields and lipid composition varied depending on the solvent composition (Fig. 3). As the water fraction increases, the percentage of product fraction (sn-2-MAG) extracted from the hexane layer increased. However, the product yield started to decrease when the water fraction reached 40% in the mixture. Therefore, we determined the mixture of ethanol/water (70/30, v/v) as the appropriate solvent fraction. At this condition, sn-2-MAG fraction with a purity of 92.4% was obtained at a yield of 78.1%, which was slightly higher than that (72.8%) obtained by silica-gel column chromatography.

The total FA compositions of fish oil substrate and sn-2-PUFA-MAG are shown in Table 1. The fish oil substrate contained over 80% PUFA at the sn-2 position, and the FA composition of sn-2-PUFA-MAG was comparable, indicating that the regioselective hydrolysis reaction was successfully performed through the Novozyme® 435-mediated ethanolysis reaction without changing the FA composition at the sn-2 position. Notably, no clear difference in the FA compositions at the sn-2 positions was observed between sn-2-PUFA-MAGs obtained via silica-gel-column chromatography and that obtained by solvent extraction method.
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3.1.4 Enzymatic preparation of sn-2-PUFA-HMFS and sn-1(3)-PUFA-HMFS

Sn-2-PUFA-HMFS and sn-1(3)-PUFA-HMFS substrates were prepared by the Lipozyme® RM-IM mediated reaction using lard, 18:1, 18:2, 18:3, sn-2-16:0-MAG rich 2-MAG substrate and/or PUFA substrates (sn-2-PUFA-MAG or PUFA-Et) (Schemes 1d and 1e). The reaction condition was similar to that of NP-HMFS with a slight modification. Notably, the dose-dependent introduction of PUFA was successfully confirmed for the preparation of PUFA-containing HMFS substrates (Fig. 4a). Almost all the PUFA reactants were located at the sn-2 position for sn-2-PUFA-HMFS substrate and at the sn-1(3) position for sn-1(3)-PUFA-HMFS substrate (Fig. 4b). The persistence of high amounts of 16:0 contents at the sn-2 position was also confirmed (Fig. 4b). Liu et al. reported that the acyl migration of 22:6 somewhat occurred time-coarsely in enzymatic interesterification by Lipozyme® RM-IM, which depends on the solvent, FA substrate, reaction temperature, and substrate molar ratio35. However, according to the report, the acyl migration did not occur at all in a short reaction time below 2 h, therefore it was expected that the use of high enzyme dosage in a short reaction time (1 h) may effectively prevent acyl migration. Actually, the location of PUFAs at the sn-2 position for sn-2-PUFA-HMFS and at the sn-1(3) position for sn-1(3)-PUFA-HMFS indicates that very little acyl migration occurred through the interesterification using Lipozyme® RM-IM (Table 2).

The lipid composition and FA composition of sn-2-PUFA-HMFS and sn-1(3)-PUFA-HMFS contained high amount of 16:0 located at the sn-2 and unsaturated FAs at the sn-1 and sn-2 positions (Table 2), which were comparable to that of HMF which has high amounts of 16:0 esterified at the TAG sn-2 position (ca. 40–60%)11, with preferential positioning of 18:1 and 18:2 at the TAG sn-1,3-positions1,2.

3.1.5 Comparison of the oxidation stability of sn-2-PUFA-HMFS with that of sn-1(3)-PUFA-HMFS

The oxidation stability of sn-2-PUFA-HMFS, sn-1(3)-PUFA-HMFS which contains 10% PUFA, and NP-HMFS was evaluated by auto-oxidation test. As shown in Fig. 5, apparently, the PV values of PUFA-containing HMFSs increased more rapidly. Because no antioxidants were present in the substrates, there was a higher susceptibility of PUFA to oxidation due to the presence of a number of activated methylene units in the molecular structure10.
3.2 Preparations of sn-1,3-18:1-sn-2-PUFA-HMFS and 1/2/3-PUFA-HMFS and their oxidation stability

Meanwhile, it is believed that the TAG containing PUFA at the sn-2 position is a better candidate than that at the sn-1 (3) position owing to properties such as better absorption characteristics as mentioned in the introduction. Considering this, we prepared sn-2-PUFA-HMFS with higher oxidation stability. Many reports have indicated that high concentration of PUFA in a TAG molecule increases the susceptibility of the TAG molecule to oxidation; the oxidation stability decreases as the number of moles of PUFA in a single TAG molecule increases. In addition, in a preliminary experiment in this study, we confirmed, using an auto-oxidation test, that the oxidation stability of sn-1 (3)-18:2-sn-2-PUFA-TAG is apparently less than that of sn-1 (3)-18:1-sn-2-PUFA-TAG (data not shown). In the following section, we report on the preparation and oxidation stability of sn-1,3-18:1-sn-2-PUFA-HMFS and 1/2/3-PUFA-HMFS.

3.2.1 Preparation via enzymatic synthesis and physical blending

We prepared PUFA-containing HMFS product, sn-1,3-18:1-sn-2-PUFA-HMFS, without highly unsaturated FA such as sn-1(3)-18:2-2-PUFA-TAG and sn-1(3)-18:3-2-PUFA-TAG in the same glycerol by physical blending with NP-HMFS (Scheme 2b). Prior to blending, sn-1,3-18:1-sn-2-PUFA-TAG substrate was prepared by enzymatic preparation using 18:1, sn-2-16:0-MAG rich 2-MAG substrate and sn-2-PUFA-MAG (Scheme 2a). In addition, 1/2/3-PUFA-HMFS was prepared by physical blending of NP-HMFS and fish oil (Scheme 2c). With regards to the FA composition of sn-1,3-18:1-sn-2-PUFA-HMFS, we confirmed that all PUFAs such as 20:5 and 22:6 were predominantly located at the sn-2 and sn-1(3) positions, respectively, and 1/2/3-PUFA-HMFS, which was prepared by physical blend, combined with PUFA without limitations (Table 2).

3.2.2 Comparison of oxidation stability

The oxidation stability of NP-HMFS, sn-1,3-18:1-sn-2-PUFA-HMFS, and 1/2/3-PUFA-HMFS substrates were evaluated by auto-oxidation test. Notably, the oxidation stability of PUFA-HMFS, which was prepared by the blending of HMFS and fish oil, was much less than that of sn-1,3-18:1-sn-2-PUFA-HMFS, in spite of the similar degree of unsaturation, indicating that the difference in oxidation stability is as a result of the difference in the molecular species (Fig. 6a). We assumed that sn-1,3-18:1-sn-2-PUFA-HMFS with excellent oxidation stability can be achieved by removing PUFA-TAG species with high degree of unsaturation in the PUFA-containing HMFS substrate.

In addition, we prepared sn-1,3-18:1-sn-2-PUFA-HMFS containing 2% PUFA at the sn-2 position, and 500 ppm of α-tocopherol (Toc), which is often removed during the preparation of enzymatic reaction and/or subsequent purification steps, was added. Without the addition of Toc, PV value rapidly increased for HMFS containing PUFA, while the addition of Toc effectively prevented increase in PV value (Fig. 6b). This means that the addition of antioxidant enhances the oxidative stability of HMFS containing PUFA at the sn-2 position.

4 Conclusion

In this study, HMFSs containing PUFA predominantly at the sn-2 position were prepared. One of the production protocols involved the preparation of sn-2-16:0-MAG and sn-2-PUFA-MAG via Novozyme® 435-mediated ethanolysis from lard and fish oil and the subsequent Lipozyme® RMIM-mediated reaction using lard, sn-2-16:0-MAG, sn-2-PUFA-MAG, 18:1, and 18:2. The other protocol involved the individual preparation of NP-HMFS and sn-1,3-18:1-sn-2-PUFA-TAG and subsequent physical blending. Comparable oxidation stability was observed with 10% similarity in the composition of PUFA at the sn-2 or sn-1(3) position. Considering physiological performance and oxidation stability, HMFS containing PUFA predominantly located at the sn-2
position is more desirable than HMFS containing PUFA predominantly located at the sn-1 (3) position.

Moreover, sn-1,3-18:1-sn-2-PUFA-HMFS showed significantly higher oxidative stability than the PUFA-containing HMFS prepared by the physical blend of NP-HMFS and fish oil substrate, demonstrating that the oxidation stability is greatly reduced by the TAG molecular species containing multiple PUFAs. The removal of PUFA-TAG molecular species with high degree of unsaturation had a significant effect. In addition, the effective enhancement of the oxidation stability of sn-1,3-18:1-sn-2-PUFA-HMFS, with PUFA located at the sn-2 position, was confirmed by the addition of Toc. Thus, the combined use of these strategies, that is, the removal of PUFA-TAG molecular species with high degree of unsaturation and the addition of antioxidants to sn-2-PUFA-HMFS, would provide an HMFS substrate with high oxidative stability and physiological performance.

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