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Human Milk Fat Substitute Produced by Enzymatic Interesterification of Vegetable Oil Blend

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Summary

Palm oil, palm kernel oil, olive oil, sunflower oil, and marine oil blend, formulated in the mass ratio of 4.0:3.5:1.0:1.5:0.2, was subjected to interesterification catalyzed by lipase from *Thermomyces lanuginosa* (Lipozyme® TL IM) for obtaining a product that contains similar triacylglycerol (TAG) structure to that of human milk fat (HMF). Reactions were carried out in a double jacketed glass vessel equipped with magnetic stirrer at 60 °C for 2, 4, 6, 8, 12 and 24 h. The blend was analyzed for fatty acid composition of both total fatty acids and those at the *sn*-2 position after pancreatic lipase hydrolysis. After interesterification, TAGs were purified by thin layer chromatography and TAG species were determined according to the carbon number (CN) by high-temperature gas chromatography. Enzymatic interesterification generated significant differences for all TAG species from CN30 to CN54. Concentrations of some TAG species (CN30, 32, 34, 36, 38, 50, 52 and 54) decreased, while some (CN40 to 48) increased after 24 h. TAG species with higher CN reached maximum levels at the end of 6 h of reaction time. The predominant TAGs of the reaction product after 24 h were CN46, 48, 50, 52 and 54 with ratios of 13.8, 18.2, 13.9, 17.8, and 12.1 %, respectively. These TAG species contain mainly 1,3-diunsaturated-2-saturated structure, like HMF.

Key words: human milk fat substitute, lipase, interesterification, triacylglycerol, fatty acid

Introduction

Enzymatic interesterification has received considerable attention in recent years. The interesterification of lipids catalyzed by lipases is an alternative to the chemical interesterification. Lipases are now widely used for the chemical redesign of fat/oil for improving physical, chemical and/or nutritional properties. With the use of lipases (EC 3.1.1.3) it is possible to produce 'tailor made' lipids for specific applications in food systems (*1–4*).

Lipids are the major source of energy in human milk or infant formulas (*5*). Hence, modification of fats and oils for infant formulas in order to obtain not only the correct fatty acid (FA) composition but also the same positional distribution as in human milk fat (HMF) *via* interesterification is being investigated. Christensen and Hølmer (*6*) prepared a HMF analogue using a *Rhizomucor miehei* lipase-catalyzed modification of butter oil. Unilever produced a milk fat substitute named Betapol for infant formulas (*7*). Also, Yang *et al*. (*8*) modified lard by lipase to produce HMF substitutes.

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Differences in chemical catalyst, non-specific lipase and *sn*-1,3 specific lipase-catalyzed interesterified products can be distinguished based on the nature of the FAs at the *sn*-2 position of the triacylglycerols (TAG) (*9*). Transesterification with *sn*-1,3-specific lipases results in the *sn*-2 FA remaining almost intact in the TAG product. This is significant from a nutritional point of view, because the 2-monoacylglycerol produced by pancreatic lipase digestion is the main carrier of FAs through the intestinal wall (*10*) and the absorption efficiency of free palmitic acid is relatively low compared to that of free unsaturated FAs (*11*). HMF contains 20–25 % of palmitic acid, and about 70 % of the FAs esterified to the *sn*-2 position of the TAG (*12*). These facts provide convincing evidence that high absorption efficiency of HMF is a result of specific structure of TAG moiety (*13*).

The objective of this study was to obtain a product which contained similar TAG structure of the HMF. For this purpose, immobilized *Thermomyces lanuginosa* lipase (Lipozyme® TL IM) catalyzed solvent-free interesterification was used to modify the blend of some vegetable oils and marine oil. The effect of reaction time on the changes of TAG species was also studied.

Materials and Methods

Materials

Palm oil (PO) was obtained from Marsa margarine factory (Adana, Turkey). Palm kernel oil (PKO) was obtained from Unilever margarine factory (Corlu, Turkey). Refined, bleached, and deodorized olive oil (OO) and sunflower oil (SFO) were obtained from the local market. ROPUFA '30' *n*-3 INF marine oil (MO) was a kind gift from Roche Vitamins Ltd., UK. Fats and oils were stored at –18 °C until use.

Lipozyme® TL IM (EC 3.1.1.3), a silica-granulated *Thermomyces lanuginosa*, which is *sn*-1,3 specific, was provided by Novo Nordisk A/S (Bagsvaerd, Denmark). Pancreatic lipase was purchased from Sigma Chemical Co. (St. Louis, MO, USA) for analysis of positional distribution of FAs in TAG. All other chemicals and reagents for the analysis were of analytical or chromatographic grade.

Enzymatic interesterification

PO, PKO, OO, SFO and MO were blended in the mass ratios of 4.0:3.5:1.0:1.5:0.2. These ratios were calculated to provide FA composition similar to that of HMF. Lipozyme® TL IM (1 g) was added to 10 g of the blend and the mixture was incubated for 2, 4, 6, 8, 12 and 24 h in a double jacketed 200-mL glass vessel. Water was circulated through the jacket from a constant temperature bath (60 °C). The mixture was stirred magnetically at 300 rpm throughout the reaction. After the reaction, the mixtures were filtered to remove the enzyme. The products were used in subsequent analysis.

Analytical methods

Determination of free fatty acids (FFAs)

FFAs were determined using a method described by Foglia *et al.* (*14*). FFAs were quantified with 0.1 M NaOH in diethyl ether/ethanol/water (3:2:2, by volume) solvent mixture (30 mL/0.5 g of sample with 4–5 drops of phenolphthalein indicator solution, 1 % in 95 % ethanol) by titrating the samples until the first traces of pink colour appeared. The percentage of FFA was expressed in terms of a FA, average molecular mass was calculated from FA composition of the blend as 243 g/mol, and calculated as follows:

$$\mathrm{FFA}=\frac{V(\mathrm{NaOH})/\mathrm{mL}\times M(\mathrm{NaOH})\times 243}{m(\mathrm{sample})/\mathrm{g}}/\% \qquad /1/$$

Isolation of TAG

A portion of the product was dissolved in hexane (1:1, by volume) and then applied (3×100 μL) in a band on a silica gel plate (Merck, Darmstadt, Germany). Plates were developed in hexane/diethyl ether/acetic acid (80:20:1, by volume), dried, and sprayed with 1 % 2,7-dichloroflourescein in methanol. To determine relative migration of the glycerides, glyceride standard (*cis*-9-monoolein, *cis*-9-1,2-diolein, *cis*-9-1,3-diolein, triolein; Supelco, Bellefonte, PA, USA) was used. The bands were visualized under an ultraviolet lamp (254 nm). The band corresponding to TAGs was scraped and extracted with diethyl ether, then the solvent was removed under a stream of nitrogen gas. The isolated TAG was used for the FA composition analysis of both total FAs and those at the *sn*-2 position.

Analysis of total FAs and *sn*-2 FAs

Fatty acid methyl esters (FAME) of TAG were prepared according to AOCS Official Method Ce 2-66 (*15*) and subsequently analyzed with HP 5890 Series II gas chromatograph (GC) (Hewlett-Packard Company, Wilmington, DE, USA) equipped with a flame ionization detector (FID) and auto sampler. A fused silica capillary column DB-Wax (30 m×0.25 mm i.d.) with a film thickness of 0.25 μm (Alltech, Deerfield, IL, USA) was used. Injection and detector temperatures were 250 and 260 °C, respectively. Oven temperature was increased from 190 to a final temperature of 220 °C at a rate of 1 °C/min. Nitrogen was used as a carrier gas at a flow rate of 1.0 mL/min. FAME mixture (37 components) and PUFA no. 1 marine oil source (Supelco, Bellefonte, PA, USA) were used to define retention times of individual FA. Results were expressed as percentage of peak area without any corrections.

Composition of *sn*-2 FAs was determined by the pancreatic hydrolysis method (*16*). TAG was mixed with 1 mL of 1 M Tris-HCl buffer (pH=7.6), 0.25 mL of 0.05 % bile salts, 0.1 mL of 2.2 % $CaCl_2$, and 8 mg of pancreatic lipase. The mixture was incubated in a water bath at 37 °C for 3 min, vortexed vigorously (1 min), centrifuged (2000 rpm, 3 min), extracted with 3 mL of diethyl ether (twice), and eluted in a sodium sulphate column. Thin layer chromatography analysis was done on silica gel 60 G plate, and developing solvent system was hexane/diethyl ether/acetic acid (50:50:1, by volume). The band corresponding to 2-monoacylglycerol (MAG) was scraped, converted to FA methyl esters, and analyzed by GC.

Determination of TAG species

Isolated TAG was also analyzed by high-temperature GC for the TAG species according to the CN. For

analysis of TAG, 0.5 μL of a solution diluted in hexane (6 mg/mL) were injected into HP 6890 GC (Hewlett-Packard Company, Wilmington, DE, USA) equipped with FID. Runs were performed from 180 to 360 °C at a rate of 3 °C/min and then isothermally at adequate time. A capillary column CP-Sil5 CB (10 m×0.32 mm i.d.) with a film thickness of 0.12 μm (Chrompack, Middleburg, The Netherlands) was used. Injection and detector temperatures were 183 and 350 °C, respectively. Helium was used as a carrier gas at a flow rate of 0.9 mL/min. Identification of the TAGs was made by comparison of retention times with those of TAG standards, *e.g.* tricaprin, tricaprylin, trilaurin, trimyristin, tripalmitin, tristearin (Supelco, Bellefonte, PA, USA). Results were expressed as area/% without any corrections. Analyses were performed in duplicate and mean values are reported.

Statistical analysis

The effects of enzymatic interesterification time on FFA values were statistically evaluated by the one way analysis of variance (ANOVA) procedure using SPSS 9.1 for Windows.

Results and Discussion

Lipozyme® TL IM is a commercial lipase and has been widely studied in the last years. Zhang *et al.* (*17*) used Lipozyme® TL IM-catalyzed interesterification of a blend of palm stearin and coconut oil for production of margarine fats. They reported that for Lipozyme® TL IM-catalyzed interesterification, at least 6 % lipase was needed to reach the equilibrium degree of interesterification in 6 h at 60 °C. They also evaluated the effect of other reaction parameters on relative degree of interesterification. When these results were taken into account, our reaction parameters were chosen as described in enzymatic interesterification section.

The system containing organic solvent is effective in the interesterification reaction, especially when the substrate is in a solid state. However, when the substrate is in a liquid state at the reaction temperature, the reaction efficiently proceeds even in a solvent free system (*18*). Therefore, we used solvent free system in our experiments.

Properties of fats and oils which were used for blend preparation are given in Table 1. PO was used as a source of palmitic acid in the blend for obtaining similar FA composition to that of HMF due to its high amount of palmitic acid (45.3 %). In addition, HMF also contains different ratios of medium and long chain FAs. In this respect, PKO, SFO, OO, and MO were used as sources of myristic, linoleic, oleic and *n*-3 (EPA and DHA) FAs, respectively, in the blend.

One of the products of lipase-catalyzed interesterification is FFA. Interesterification reaction usually occurs together with hydrolysis. Formation of FFAs at different interesterification times is shown in Fig. 1. Water content of immobilized enzyme caused a sharp increase of FFAs at the end of 2-hour reaction. The FFA content reached equilibrium within the first two hours. Differences in the FFA content between 2- and 12-hour reactions were found insignificant ($p>0.05$), while 24-hour reaction caused a significant change. This may be due to evaporation of water from the mixture into atmosphere of the reactor. After a 12-hour reaction, esterification became dominant reaction compared to the hydrolysis.

Table 1. FA composition (%) of fats and oils used in the blend (mean values, *N*=2)

FA[a]	PO	PKO	SFO	OO	MO	Blend
C6.0		0.3				0.1
C8:0		3.6				1.5
C10:0	0.1	3.5				1.3
C12:0	0.2	48.2				17.8
C14:0	1.1	16.2	0.1		4.1	6.0
C15:0	0.1				1.2	
C16:0	45.3	8.5	6.0	11.2	21.1	23.0
C16:1	0.2		0.2	0.9	5.3	0.3
C17:0	0.1			0.1	1.8	
C17:1				0.1	0.8	
C18:0	4.2	2.1	3.2	2.5	5.5	2.5
C18:1	39.3	15.0	33.4	73.7	12.8	31.9
C18:2	8.6	2.6	55.8	10.2	1.3	14.0
C18:3	0.1		0.1	0.5	0.6	0.1
C20:0	0.4	0.1	0.2	0.4	0.5	0.2
C20:1	0.1	0.1	0.2	0.3	0.8	0.1
C20:5					7.0	0.1
C22:0	0.1		0.6	0.1	0.4	0.1
C22:6					35.5	0.7
C24:0	0.1		0.2	0.1	1.5	0.1
FFA/%	0.35	0.25	0.40	0.69	0.09	0.35

[a]FAs are designated by number of carbon atoms/number of double bounds

FA: fatty acid residues; PO: palm oil; PKO: palm kernel oil; SFO: sunflower oil; OO: olive oil; MO: marine oil; FFA: free fatty acid

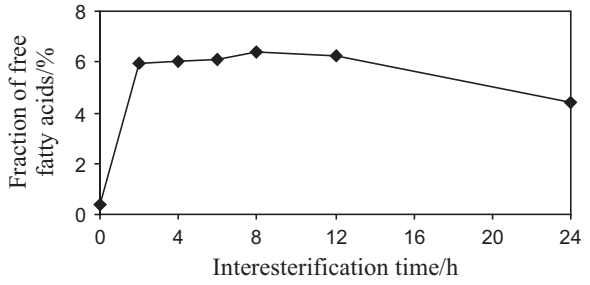

Fig. 1. Free fatty acids formation during the different interesterification times

Lipozyme® TL IM is an *sn*-1,3 specific lipase. Therefore the FAs originally located at the *sn*-2 position should largely remain in this position even though some degree of acyl migration into *sn*-1,3 position might occur (*17*). The FA profiles of both total FAs and those at *sn*-2 position of the blend are shown in Table 2. The main FAs of the HMF are also given in the same table. Major FA residues observed in the blend were: lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1), and linoleic acid (C18:2), like in HMF. Lauric acid was found at higher percentage (17.8 %) in the blend than in

Table 2. Positional distribution (%) of fatty acids of oil blend and human milk fat (mean values, *N*=2)

FA	Blend[a]				HMF[b]			
	total	*sn*-2	*sn*-2[c]	*sn*-1,3[d]	total	*sn*-2	*sn*-2[c]	*sn*-1,3[d]
C12:0	17.8	13.9	25.9	19.8	4.9	5.3	36.0	4.7
C14:0	6.0	5.6	31.2	6.2	6.6	11.2	57.0	4.3
C16:0	23.0	28.7	41.5	20.2	21.8	44.8	68.0	10.3
C18:0	2.5	4.5	59.6	1.5	8.0	1.2	5.0	11.4
C18:1	31.9	34.8	36.4	30.5	33.9	9.2	9.0	46.3
C18:2	14.0	10.6	25.2	15.7	13.2	7.1	18.0	16.3

[a]C6:0, C8:0, C10:0, C16:1, C18:3, C20:0, C20:1, C20:5, C22:0, C22:6, and C24:0 are present in minor proportions at the *sn*-2 and *sn*-1,3 positions in the blend
[b]Values are derived from Lien *et al.* (*19*)
[c]Indicates the percentage of the fatty acids esterified at the *sn*-2 position, calculated as [*sn*-2 FAs×100/(3×total FAs)]/%
[d]Indicates FA composition at the *sn*-1,3 positions, calculated as [3×total FAs–(*sn*-2)]/2

HMF. The original content of C16:0 in the *sn*-2 position of the blend was lower than the amount of HMF. However, the level was much higher than that found in most ordinary infant formulas today. The FA composition at *sn*-1,3 positions of enzymatically modified blend is given in Table 2. It can be seen that *sn*-1,3 positions predominantly contain unsaturated FAs, mostly oleic acid, like in HMF, except for lauric acid.

Percentage of total unsaturated FAs of the blend (47.3 %) is nearly equal to that of HMF. Diets high in unsaturated FAs are advantageous in infant feeding, since they are absorbed better than saturated FAs due to less interference with calcium absorption (*20*).

The TAGs can be grouped according to their CN, which is the number of carbons in the constituent FA. TAG species were determined at the end of 2, 4, 6, 8, 12 and 24 h of the reaction. Table 3 shows the changes in the percentage of the TAG species of the blend at different reaction times according to the CN. The lipase--catalyzed interesterification of the blend caused many changes in the TAG species. It was considered that the starting blend contained predominately CN52 and 54 TAG species from the SFO and OO and medium chain FA TAG species (CN32 to 38) from the PO and PKO. Interesterification of the blend in the presence of an *sn*-1,3 specific lipase resulted in the incorporation of unsaturated FA into 1- and 3-positions of the glycerol. As a result of these changes, the relative percentage of several TAG species (CN40, 42, 44, 46 and 48) increased, while others (CN30, 32, 34, 36, 38, 50, 52 and 54) decreased. However, a significant increase was observed between 6 and 8 h of reaction. The yield of CN46 and 48, which include mainly palmitic acid, increased 7- and 3-fold after the 6-hour reaction, respectively. Similar results were reported by Macrae (*21*).

Table 3. Carbon number (CN) and triacylglycerol percentage of the blend after different times of lipase-catalyzed interesterification (mean values, *N*=2)

CN	Time/h						
	0	2	4	6	8	12	24
	TAG/%						
30	0.3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
32	1.9	0.3	0.2	0.2	0.3	0.2	0.2
34	2.7	0.4	0.3	0.3	0.5	0.3	0.3
36	7.3	2.2	1.8	1.4	1.8	1.4	1.4
38	5.4	2.5	2.4	2.2	2.6	2.3	2.0
40	3.2	3.5	3.5	4.0	4.2	4.2	3.7
42	3.4	7.1	7.4	8.6	8.4	8.5	7.7
44	2.3	7.1	7.5	8.7	8.3	8.0	8.1
46	2.0	12.3	12.8	14.7	14.1	13.9	13.8
48	6.2	15.6	16.8	18.7	17.4	18.4	18.2
50	18.2	15.3	14.8	12.8	13.4	13.4	13.9
52	23.2	20.2	19.4	16.6	17.2	17.0	17.8
54	22.8	12.5	12.3	11.1	11.1	11.8	12.1
Others	1.2	1.1	0.8	0.7	0.7	0.6	0.8

Variation of each TAG species according to the CN with different reaction times was calculated as follows:

$$X = \frac{P_{t1} - P_{t0}}{P_{t0}} \qquad /2/$$

where P_{t1} is the peak area of TAG at any time of reaction and P_{t0} is the peak area of TAG of the blend.

The changes for the increasing TAG species of the blend after different interesterification times are illustrated in Fig. 2. Maximal variation was obtained in the majority of TAG species between 0 and 6 h of reaction time. However, no further changes in variation could be achieved at longer reaction times. Similar changes were observed for variations of decreasing TAG species (data not shown). For this reason, it can be concluded that 6 h of reaction time is enough for optimum conversion.

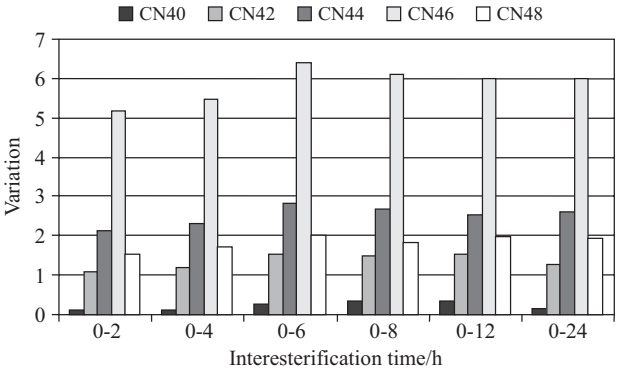

Fig. 2. Change of the increasing triacylglycerol species of the blend with different interesterification times

The predominant TAGs were CN46 (13.8 %), CN48 (18.2 %), CN50 (13.9 %), CN52 (17.8 %), and CN54 (12.1 %) for the blend enzymatically modified for 24 h. Total percentage of these TAG species in HMF was 83 % (*22*). When the compositional distribution of the blend was taken into account, it may be assumed that these TAG species contain mainly 1,3-diunsaturated-2-saturated structure, like HMF.

Conclusion

FA composition of the interesterified blend resembles to that of HMF, especially *sn*-1,3 positions predominantly contain unsaturated FAs, mostly oleic acid. However, the distribution of FAs at the *sn*-2 position is a little different; the proportion of palmitic acid is not at a desired level. But still, the study has demonstrated that enzymatic interesterification (with 1,3-specific lipase) of an oil blend containing fats and oils with a high amount of palmitic acid at *sn*-2 position can be used in a process for obtaining HMF substitutes. It appears that interesterification reactions described herein may not be optimal for a given application; additional technological treatments, *e.g*. fractional crystallization, can increase the proportion of palmitic acid at *sn*-2 position. Enzyme-catalyzed interesterification of fats and oils may be an alternative to physical blending or chemical interesterification for producing HMF substitutes.

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