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Synthesis of novel medium-long-medium type structured lipids from microalgae oil via two-step enzymatic reactions

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ABSTRACT

The aim of this study is to produce new medium-long-medium structured triacylglycerols (MLM-STAGs) containing caprylic acid (CA) at the *sn*-1,3 positions, and long chain saturated fatty acids (LCSFAs) and bioactive fatty acids at the *sn*-2 position via two-step enzymatic process by Lipozyme TL-IM, using autotrophic microalgae (*Isochrysis galbana*) TAGs. In the first step, the ethanolysis of microalgae oil TAGs for 2-monoacylglycerols (2-MAGs) production are performed in the presence of water content of 5 wt% at ethanol/oil mole ratio of 24:1 with Lipozyme TL-IM at 25 °C for 12 h. In the larger-scale production of 2-MAGs, the 2-MAGs yield and the purity of the purified MAGs attained are 31.44-36.08 wt% and 91.57 mol%, respectively. In the second reaction, these 2-MAGs are esterified with caprylic acid (CA) in *n*-hexane system to synthesize almost pure MLM-STAGs with the recycled Lipozyme TL-IM under the optimized conditions. The final MLM-STAGs contain 64.28% CA and 13.35% ω-3 polyunsaturated fatty acids. The CA-LCSFAs-CA (43.01%) and novel CA-stearidonic acid (SDA, $C_{18:4\omega-3}$)-CA (23.88%) are the major MLM-STAGs present. In all, *Isochrysis galbana*-derived oil containing SDA is a promising source for producing high-value microalgal bioproducts by two-step enzymatic process in a cost-effective manner.

1. Introduction

Structured lipids (SLs) are generally known as structured triacylglycerols (STAGs) that have been chemically or enzymatically developed to incorporate the desired fatty acids into specific position of the glycerol backbone for nutritional and therapeutical applications [1,2]. Many clinical and nutritional observations have shown that SLs can be used for treating or preventing particular human diseases [3,4], as well as to modify the physiochemical characteristics of lipids [5]. To produce SLs with unique properties, desired fatty acids, including short-chain fatty acids (C_2-C_6), medium-chain fatty acids (MCFAs, C_8-C_{12}) and long-chain fatty acids (LCFAs, $\geq C_{14}$), are incorporated into specific positions of glycerol [6–9]. Typical SLs of the best-known medium-long-medium STAGs (MLM-STAGs) contain MCFAs at the *sn*-1 and *sn*-3 positions of the glycerol backbone and long-chain saturated fatty acids (LCSFAs, $\geq C_{14}$) or essential fatty acids (EFAs) at the *sn*-2 position, exhibiting maximum health-beneficial and nutritive properties [7,10]. After hydrolyzed by pancreatic lipase (*sn*-1,3 specific lipase), the liberated MCFAs are absorbed in the intestinal cell and rapidly metabolized through mitochondrial β-oxidation in the liver cell to provide energy [2,11]; the 2-monoacylglycerols (2-MAGs) of fatty acids at the *sn*-2 position are effectively incorporated into chylomicrometers and readily absorbed through the lymphatic system [1,2]. Mu and Høy have found that the feeding of MLM-STAGs can modify the TAGs species of mice lymph to promote the absorption of fatty acids at *sn*-2 position of MLM-STAGs compared with LLL-STAGs and MLL-STAGs [12]. Zhou et al. have shown that dietary MLM-STAGs can reduce body weight of the obese C57BL/6L mice by regulating lipid metabolism and modifying the effect of gut microbiota [13].

Compared with chemical method, enzymatic method has been widely used in preparing MLM-STAGs products in view of mild conditions, high catalytic specificity, no toxicity and oxidation, easy separation, and so on [5,14–17]. Using an enzyme as biocatalyst, strategies for MLM-STAGs production include one-step and multistep (two-step and three-step) enzymatic process [15]. One-step enzymatic approach for MLM-STAGs production has the following disadvantages, such as the difficult purification to collect the desired SLs from unreacted TAGs and some side products (e.g. diacylglycerols (DAGs) and

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MAGs), the low specific FAs incorporation rate (< 50%), and the high acyl migration rate [9,15]. Nevertheless, three-step enzymatic process for SLs involves esterification, glycerolysis and acidolysis by three lipases (immobilized *Psuddomonas cepacia* lipase, Novozym 435 and Lipozyme RM-IM) using palm oil as the starting substrate [15,18]. In fact, three-step enzymatic process is difficult to be successfully scaled up in an industrial application, due to the time-consuming, high cost, labor-intensive and environmental-unfriendly procedures [15]. To address these drawbacks, the so-called two-step enzymatic approach is an interesting method [6–8,15]: i) yielding 2-MAGs by enzymatic ethanolysis of natural oils using a *sn*-1,3 specific lipase; ii) obtaining pure MLM-STAGs via lipase-catalyzed esterification of purified 2-MAGs and MCFAs.

Extensive studies have focused on structurally modified lipids by lipase-catalyzed reaction of traditional oils such as vegetable and animal oils [7,9,19,20]. However, vegetable oils do not contain long-chain ω-3 polyunsaturated fatty acids (LC ω-3 PUFAs) like eicosapentaenoic acid ($C_{20:5\omega-3}$, EPA) and docosahexaenoic acid ($C_{22:6\omega-3}$, DHA) [9,19,20]. Although fish oil is a major source of LC ω-3 PUFAs for human consumption [21], an increasing price (around 2400 USD/ton) and a strong fishy odor affect the increasing demand of human consumption [22]. Currently, the search for new sources of oil aimed at the production of SLs, is ongoing in order to maximize both nutritive value and economic benefit [22–24]. Recently, oleaginous microalgae are proven to be one of the most potential and sustainable alternatives to PUFAs of fish oil in the future, since they are capable of accumulating large amounts of lipids and can be easily cultured at an industrial scale [22,24–26]. Among the investigated marine microalgae species, *Isochrysis galbana* (*I. galbana*) is regarded as one of the potential oleaginous model microalgae, due to the high CO_2 fixation rate, great photosynthetic efficiency, high lipid yield and PUFAs productivity, and no cell wall for easy extraction of oil [22,25,26]. Moreover, *I. galbana*-derived biomass has been incorporated into some traditional products (e.g. biscuits and pasta) for nutraceutical (ω-3 PUFAs) [27,28]. These evidences indicate that *I. galbana* oil as an alternative to ω-3 PUFAs of fish oil can be safe for human consumption [24].

Lipozyme TL-IM (lipase from *Thermomyces lanuginosus* (lipase TL) is immobilized on granulated silica) is a *sn*-1,3 specific lipase [14], with a cheaper price in comparison to the other commercial immobilized biocatalysts such as Novozym 435 and Lipozyme RM-IM [14,29]. Our previous study has shown that lipase TL can catalyze fish oil to produce the highest yield of MAGs through ethanolysis in comparison to CALA (lipase A from *Candida antarctica*) and CALB (lipase B from *Candida antarctica*) [30]. Additionally, like most of lipases, lipase TL is an active biocatalyst to esterify glycerol and free fatty acid for MAGs and DAGs formation [14,31]. These findings suggest that lipase TL is a right candidate for MLM-STAGs production by the two-step enzymatic process (ethanolysis and esterification). Thus, this study aims to produce MLM-STAGs containing caprylic acid (CA) at the *sn*-1,3 positions and LCSFAs and EFAs at the *sn*-2 position via two-step enzymatic process by Lipozyme TL-IM using *I. galbana*-derived TAGs. Reaction conditions of two-step enzymatic process, such as water content, substrate mole ratio, temperature and reaction time, were investigated. Furthermore, the characteristics of the products were analyzed with respect to the composition and positional distribution of fatty acids in the MLM-STAGs, and melting and crystallization properties.

2. Materials and methods

2.1. Materials

galbana cells were cultivated, collected and dried as described in our previous study [32]. The composition and regiodistribution of fatty acid in purified TAGs from microalgae oil were determined in Table 1. Lipozyme TL-IM (specific activity, 50,000 U/g) was kindly donated by Novozymes A/S (Bagsvaerd, Denmark). All solvents, analytical reagents and the standards of different lipid class were obtained from Sigma Aldrich Co (St. Louris, MO).

Table 1
The composition and positional distribution of fatty acids of *I. galbana* TAG, and fatty acid composition of MAGs fraction obtained by ethanolysis with Lipozyme TL-IM under the optimal conditions.

Fatty acid	Microalgae TAGs (Mass ≈ 854)	*sn*-2 position of microalgae TAGs	MAGs product by ethanolysis [a]
C14:0	16.13 ± 1.24	22.93 ± 1.77	20.80 ± 1.79
C16:0	14.81 ± 0.74	24.27 ± 1.42	22.26 ± 1.34
C18:0	1.60 ± 0.33	0.55 ± 0.11	0.37 ± 0.06
ΣLCSFAs	32.54 ± 0.91	47.75 ± 1.46	43.41 ± 1.72
C14:1	0.86 ± 0.13	1.80 ± 0.32	0.91 ± 0.27
C16:1	2.35 ± 0.17	3.08 ± 0.25	1.54 ± 0.15
C18:1	27.95 ± 1.16	4.38 ± 0.38	3.74 ± 0.85
ΣMFAs	31.16 ± 0.58	9.26 ± 0.29	6.19 ± 0.47
C18:2ω-6	4.17 ± 0.22	5.84 ± 0.50	5.80 ± 0.51
Σω-6 FAs	4.76 ± 0.10	6.58 ± 0.44	6.21 ± 0.62
C18:3ω-3	5.54 ± 0.28	6.14 ± 0.47	6.92 ± 0.63
C18:4ω-3	13.61 ± 0.59	23.44 ± 0.76	25.16 ± 2.84
C20:4ω-3	0.82 ± 0.30	0.47 ± 0.15	0.95 ± 0.04
C20:5ω-3	0.68 ± 0.14	0.61 ± 0.14	1.17 ± 0.28
C22:6ω-3	5.71 ± 0.18	3.59 ± 0.21	6.91 ± 0.63
Σω-3 PUFAs	26.36 ± 0.48	34.25 ± 0.63	41.11 ± 1.74
Σ EFAs	31.12 ± 0.61	40.83 ± 0.80	47.32 ± 1.42
ΣLCSFAs + EFAs	63.66 ± 0.72	88.58 ± 0.76	90.73 ± 0.97

[a] MAGs were obtained by ethanolysis under the optimum conditions (in the Section 3.1).

2.2. Microalgae oil extraction and TAGs purification

Microalgae oil was extracted by Soxhlet method using *n*-hexane as organic solvent [33]. The crude microalgae oil was used to separate and purify microalgae TAGs by silica gel column according to the method of Bondioli et al. [34]. The organic solvents were removed by a rotary evaporator (RV 10 digital, IKA, Germany). The acid, saponification, iodine, peroxide and *p*-anisidine values of microalgae TAGs were 0.82 mg KOH/g, 193.68 mg KOH/g, 82.35 I_2/100 g, 1.72 mmol/Kg and 0.47, respectively. Official methods of the American Oil Chemist's Society were used to determine the acid (Cd 3d-63), saponification (Cd 3-25), iodine (Cd 1-25), peroxide (Cd 8-53) and *p*-anisidine (Cd 18-90) values [35]. The purified microalgae TAGs were stored at −20 °C for analysis and enzymatic reaction.

2.3. Two-step enzymatic reactions

2.3.1. Ethanolysis of microalgae TAGs for 2-MAGs production

A reaction mixture consisted of microalgae TAGs (1 g), anhydrous ethanol, distilled water and immobilized lipase (10% by weight of oil) were added to dark-colored and screw-capped flasks (25 mL) [7]. The reaction system was connected in a thermostatic water bath equipped with a magnetic stirrer. Reaction temperature was controlled at 25 °C and stirring speed was set at 300 rpm. Different mole ratio of ethanol to oil (2:1–40:1) and water content (0–13%, wt%) were studied. At the end of the experiments (6 h), the samples were withdrawn and centrifuged at 5000 rpm for 10 min to remove the biocatalyst. All samples were stored at −20 °C for further analysis.

To reduce the cost, the used lipase loading was reduced and the reaction time was prolonged to 12 h when larger scale reaction was performed. The ethanolysis of oil catalyzed by Lipozyme TL-IM for 2-MAGs product was scaled up in the following conditions: 20 g microalgae TAGs, 25.85 g anhydrous ethanol, 5 wt% water content, 1 g lipase loading, reaction time 12 h. The reaction was performed in a 250-mL stirred tank reactor. The jacketed reactor was connected with a circulated water bath to maintain the constant reaction temperature (25 °C). Stirring was provided by a magnetic stirring apparatus. Oils, anhydrous ethanol and extra water were firstly added to the reactor, followed by