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Enrichment of n-3 polyunsaturated fatty acids into acylglycerols of borage oil via lipase-catalyzed reactions under supercritical conditions

Tsao-Jen Lin *, Shin-Wan Chen

Department of Chemical Engineering, National Chung-Cheng University, Chia-Yi 621, Taiwan

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Abstract

Esterification of free n-3 polyunsaturated fatty acid (PUFA) with enriched n-6 triglyceride (TG) was catalyzed to modify the desired structured lipid with n-3/n-6 = 4 by using lipase under supercritical carbon dioxide ($SCCO_2$). Comparing four different types of lipases, 1,3-specific lipase from *Mucor miehei* has the highest degree of incorporation under 10 wt.% loading amount of total substrates. The optimal operating parameters under 10.2 MPa and 323.15 K $SCCO_2$ can attain the desired n-3/n-6 ratio in 6 h. Because of the negative effect on the enzyme activity by the enriched n-6 TG, the optimal substrate ratio of the enriched n-6 TG and the n-3 PUFA was chosen as 1/4. To enhance the solubility of n-3 PUFA in $SCCO_2$, ethanol was applied as a co-solvent and reached an optimal input at 10 wt.% of total substrates. The activity of the enzyme still maintained 81% of initial activity because of de-moisture from the surface enzyme after seven cyclic pressurization/depressurizations. A model of the ping-pong mechanism and the substrate inhibition was proposed to express the catalytic action of lipase. With the aid of the Michaelis–Menten equation and Lineweaver–Burk plot, the estimated reaction rate has a deviation of only 2.11% from the experiment data.

Keywords: Supercritical fluid; n-3 polyunsaturated fatty acid; Trans-esterification; Production kinetics; Enzyme technology

1. Introduction

Long-chain polyunsaturated fatty acids (i.e., n-3 and n-6 PUFA) are important components of membrane phospholipids in microglia, neurons, and immune cells [1,2]. Gamma-linolenic acid (GLA, 18:3n-6; all-*cis*-6,9,12-octadecatrienoic acid) is an essential fatty acid (EFA) in the n-6 family primarily found in plant-based oils, and is an essential intermediate in the normal metabolism of linolenic acid (18:2n-6) to the eicosanoid precursor arachidonate [3,4]. GLA could increase tissue biosynthesis of series-1 prostaglandins and plays an important role in treatments, including diabetes, hypertension, thromboembolic disease, and in the regulation of inflammatory response [5-7]. However, excess amounts of GLA used long term can actually increase inflammatory, platelet and vasoconstriction problems if it is not balanced with sufficient n-3 PUFA [8]. The reason for this is because n-3 PUFA can influence series-2 eicosanoid concentrations by competitively inhibiting the breakdown of GLA by cyclooxygenase [9]. In fact, for optimum health, several studies reported that the optimal ratio of n-3 to n-6 fatty acids is 4 [10]. Both *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA, 20:5n-3) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA, 22:6n-3) are EFAs in the n-3 family and are metabolized from α-linolenic acid (18:3n-3).

The metabolic processes on GLA, EPA, and DHA, consist of a series of alternating steps of desaturation and elongation, with desaturation being the rate-limiting step. The Δ6 desaturase can be impaired by several factors, such as aging, high levels or alcohol or cholesterol, and cancer and virally infected cells [11]. Furthermore, because they are easily oxidized to become hydro-peroxides, these PUFAs are usually preserved in the form of triglycerides (TG) or ethyl ester. Lawson and Hughes [12] reported that human absorption of PUFA is better as TG than as ethyl ester. Recently, many attempts have been carried out in trans-esterification or inter-esterification reactions to modify and enrich the content of n-3 PUFA in vegetable oil, melonseed oil, borage oil, and evening primrose oil by using various lipases as biocatalysts [13–16]. It was found that lipases were efficient in these reactions because of their high regiospecificity and mild reaction conditions within low temperatures and organic solvents. However, the drawbacks of the organic solvents used as reaction media are long time energy consumption, and numer-

* Corresponding author. Tel.: +886 5 2720411x33405; fax: +886 5 2721206.
E-mail address: chmtjl@ccu.edu.tw (T.-J. Lin).

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ous environmental and residual solvent issues caused by the purification of products.

Supercritical fluids (SCFs) used as a medium have been employed to conduct enzymatic reactions and to shorten the reaction time. The higher diffusivity and lower viscosity compared to organic solvents make SCFs more attractive as a reaction medium or carrier fluids for transporting reactants and products in several processes, such as extraction, separation, and reaction. Furthermore, the solubility of solute in SCFs is strongly associated with the density, which can be easily controlled by variation of temperature and pressure. Carbon dioxide (CO_2) as a safe SCF solvent medium is of particular interest to the food and pharmaceutical industries, because of its non-toxicity and non-flammability [17,18]. CO_2 can also offer a non-oxidative environment and is at a critical temperature (31.05 °C) that is suitable for processing thermal sensitive materials. However, supercritical carbon dioxide ($SCCO_2$) has a low dielectric constant and polarizability, which make many strong polar compounds in $SCCO_2$ have very low solubility. Adding a small amount of polar materials (e.g., water, methanol, ethanol, and acetone) as co-solvents into $SCCO_2$ can significantly enhance the solubility [19,20]. Several possible mechanisms of co-solvent effects on the solubility are attributed to: (i) chemical interactions, such as hydrogen bonding or formation of charge transfer complex between solute and co-solvent; (ii) physical interactions, such as dipole–dipole coupling between co-solvent and solute; (iii) the increasing density of a SCF solution [20].

The mechanism of the esterification of essential fatty acids on to TG with lipase is very complicated owing to its involving multi-reactants and multi-products in the reaction system. Generally, there are three kinds of mechanisms to account for this multisubstrates–enzyme reaction system, such as the ping-pong bi-bi mechanism, ordered mechanism, and random order mechanism [21,22]. In the past, most extended models for the trans-esterification were based on the application of Michaelis–Menten assumptions [23,24]. This type of model seems to be valid for the most enzymatic reactions. In this work, we examined the incorporation of free n-3 PUFA, derived from the saponification of menhaden oil, into the enriched-GLA (n-6) TG of borage oil by an immobilized lipase catalyzing esterification under $SCCO_2$. The effects of lipase, reacting time, system pressure and temperature, co-solvent, and substrate ratio on production of the structured lipid in optimal ratio (n-3/n-6 = 4) were investigated. The kinetic characteristics of the reaction under $SCCO_2$ would be investigated and the rate expression described by a modified Michaelis–Menten equation as well.

2. Experimental setup

2.1. Materials

Immobilized lipases (including *Pseudomonas* sp., *Candida rugosa*, and *Candida antarctica*), menhaden oil, and borage oil were purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized 1,3-specific lipase (Lipozyme IM-60) from *Mucor miehei* was a gift of Novo Nordisk Bioindustry Co. Ltd. Heptadecanoic acid, *cis*-6,9,12,15-octadecatetraenoic acid methyl ester (C18:4), γ-linolenic acid methyl ester (C18:3), eicosapentaenoic acid methyl ester (C20:5), and docosahexaenoic acid methyl ester (C22:6) for calibration curves of gas chromatography (GC) analysis were purchased from Sigma Chemical. Other chemical reagents, for example, trimethylsulfonium iodide, ethanol, *n*-hexane, methanol, 1,2-dichloroethane, NaOH, Na_2EDTA, HCl, urea, silver oxide, and molecular sieve, were obtained from J. T. Baker (U.S.A.) and Riedel-de Haen (Germany).

Table 1
PUFA composition (wt.%) of menhaden oil before and after urea inclusion method

Fatty acids	Before	After
C14:0	4.7	1.2
C16:0	18.7	2.3
C16:1, *cis*-9	17.5	3.2
C18:0	3.1	0.2
C18:1, *cis*-9	12.9	2.2
C 18:4, n-3	2.6	6.3
C20:5, n-3	16.1	21.7
C22:6, n-3	17.7	52.3
Total n-3	36.4	80.3

2.2. Free n-3 PUFA preparation

The preparation of free n-3 PUFA from menhaden oil was carried out by urea inclusion method [25]. Basically, the urea inclusion method includes saponification, formation of urea inclusion complex, and extraction of free n-3 PUFA. The final concentrated n-3 PUFA was stored under −25 °C. Table 1 shows the compositions of free PUFA after urea inclusion method. Total n-3 PUFA content is 80.3 wt.%, in which contains 74 wt.% EPA and DHA.

2.3. Enrichment of GLA on TG

The reaction was carried out by a lipase-catalyzed selective partial hydrolysis of borage oil [26]. Lipozyme IM-60 was used as the lipase for this reaction. The reaction mixture contained borage oil (50 mg) and 5 mg IM-60 in 1 mL water was incubated in an Erlenmeyer flask at 20 °C for 2 h. Then, the reacted mixture was extracted three times with hexane. Analyzing the percentage of different TG, Table 2 shows that the mono-TG increases from 28.9% to 80.5%. Besides, the content of GLA in TG was increased from 18.3% to 32.1%, as shown in Table 3.

Table 2
TG composition (wt.%) of borage oil before and after lipase-catalyzed selective partial hydrolysis

Lyceride	Before	After
Glycerol	2	3
Mono-	28.9	80.5
Di-	31.1	9.5
Tri-	38	7

Table 3
PUFA composition (wt.%) of borage oil before and after lipase-catalyzed selective partial hydrolysis

Fatty acids	Before	After
C16:0	12.1	10.8
C18:0	4.8	2.3
C18:1	19.9	17.5
C18:2	39.1	34.4
C18:3, n-6	18.3	32.1
C20:1–C 24:1	5.8	2.9

2.4. Esterification under $SCCO_2$

In this study, $SCCO_2$ was used as solvent for esterification between the enriched-GLA TG in borage oil and the prepared free n-3 PUFA as reactants. The experimental apparatus is shown in Fig. 1. A high-pressure reactor, which can tolerate up to 40 MPa with an inside volume of 400 mL, was used to carry out the reaction. The reactor along with a stirrer to completely mix up substrates and enzyme was placed in an isothermal water bath (Wisdom, model LC-06) to control the reaction temperature within ±0.05 K. The temperature was measured in the high-pressure cell with a NiCr–NiAl thermocouple (Thermocoaxe type K) in contact with the measuring fluid and the thermocouple was calibrated with a platinum thermometer (Guildline, model 9540). The temperature was measured to an accuracy of within ±0.01 K. The pressure measurement was carried out by a 50.4-MPa pressure transducer (Sensotec, model AG-300) with an accuracy ±0.05 MPa. Liquid carbon dioxide through a cooling unit and a layer of molecular sieve to remove the extra water was pressurized with a high-pressure pump (ISCO 500D Model Syringe pump) and stored in a buffer container. To prevent from running short of CO_2 during operation, the buffer container was used as a pressure source to supply the higher-pressure liquid CO_2. Meanwhile, in order to enhance the solubility of substrates, ethanol was used as co-solvent. The reactants were pre-mixed with ethanol before being pumped into the reactor by a liquid pump (Shimadzu LC-8A). A specific amount of enzyme was pre-deposited in the reactor before the system was pressurized. After reaction, products released by a metering valve were collected in hexane within an ice bath collector. Then, the content of fatty acids on TG was analyzed with gas chromatography (Shimadzu GC-17A).

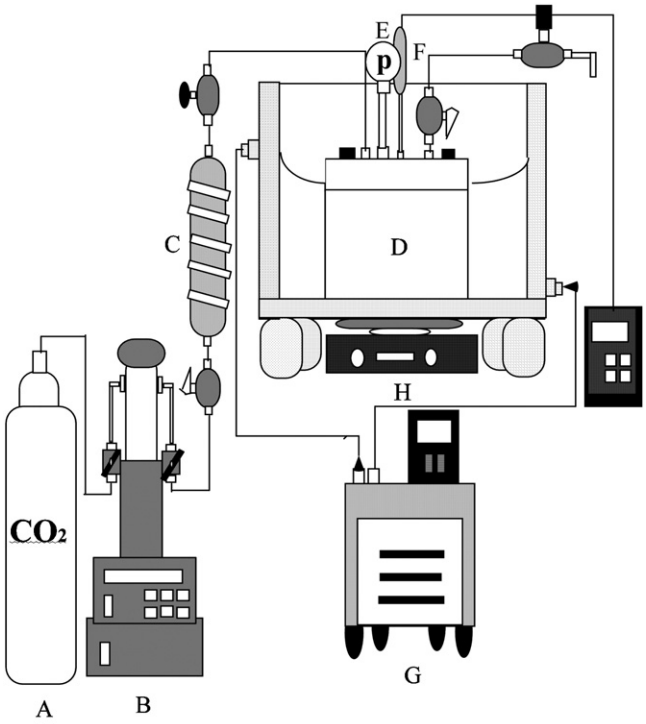

Fig. 1. Schematic of the experimental facility.

2.5. Analysis of fatty acid composition on TG by GC

First, sodium methoxide was added into the product oil to deposit the free fatty acids. Then, the heptadecanoic acid with chloroform solution was added as internal standard in the sample. In order to convert the fatty acids on TG into fatty acid methyl ester, 50 μL of 1,2-dichloroethane and 50 μL of 0.2 M trimethylsulfonium hydroxide (TMSH) methanol solution were added [27]. The GC column (30 m × 0.25 mm id., 0.1 μm film thickness) is the fused silica capillary column Rtx-65 TG from Restek company. GC setting conditions were as follows: injection temperature 250 °C, flame ionization detector (FID) temperature 250 °C, flow rate of carrier gas N_2 1.2 mL/min, and oven temperature 170 °C (hold 1 min.) to 250 °C with a rate of increase 3 °C/min. The concentration of fatty acid esters was determined from the calibration curves by the measured peak area ratio.

2.6. Enzymatic stability in $SCCO_2$

Trans-esterification was carried out in a run under 10.2 MPa and 50 °C $SCCO_2$ for 6 h, enriched n-6 TG/n-3 PUFA = 1/4, and enzyme and co-solvent content 10 wt.% of total substrates, respectively. Every 2 days, the reaction was executed and the residual lipase activity was measured. The residual activity is defined as the ratio of the conversion on the *n* run to that on the initial run. Every point represents the average of three experiments.

3. Results and discussion

3.1. Lipase effect

Two types of immobilized lipases, including 1,3-specific lipases (i.e., *M. miehei* and *Pseudomonas* sp., which specialize to prepare the designated TG by selective addition of free PUFA into the 1.3-positions of TG) and nonspecific lipases (i.e., *C. rugosa* and *C. antarctica*, which randomly incorporate free PUFA into any position of TG), were to incorporate free n-3 PUFA into the enriched n-6 TG of borage oil at 10.2 MPa

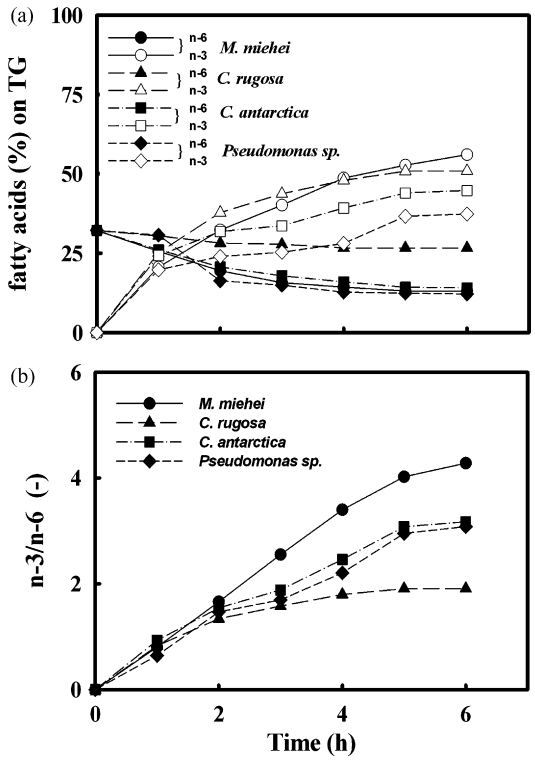

Fig. 2. The time course of (a) total n-6 and n-3 contents and (b) ratio of n-3 to n-6 for four different immobilized lipases, including *M. miehei*, *C. rugosa*, *C. antarctica*, and *Pseudomonas* sp., under 10.2 MPa 323.15 K $SCCO_2$, enriched n-6 TG/n-3 PUFA = 1/4, and enzyme and co-solvent loadings 10 wt.% of total substrates, respectively.

and 323.15 K $SCCO_2$, as shown in Fig. 2. After 6-h reaction, the degree of n-3 PUFA incorporation attained with the various lipases was in the order of *M. miehei* > *C. rugosa* > *C. antarctica* > *Pseudomonas* sp. These results were different from those reported by Senanyake and Shahidi [28], wherein the degree of DHA incorporation into borage oil in hexane at ambient was in the order of *C. antarctica* > *Pseudomonas* sp. > *M. miehei* > *C. rugosa*. According to Zagrobelny and Bright [29], the protein conformations of the local active sizes on enzymes have different responses to pressure, even in the same type of specific lipases. The nonspecific lipase of *C. rugosa* was effective in corporation of n-3 PUFA, however it was ineffectual in hydrolysis of n-6 fatty acids out off TG and leading to the lowest ratio of n-3/n-6. From Fig. 2(a and b), the 1,3-specific lipase from *M. miehei* gave the highest degree of incorporation (56%) and reached the optimal ratio of n-3/n-6 after 6 h. Hence, by considering the degree of incorporation and the time to reach the optimal ratio of n-3/n-6, this lipase was selected for subsequent experiments to determine optimal esterification under supercritical conditions.

The effect of lipase loading on the incorporation of free n-3 PUFA into the enriched n-6 TG of borage oil was shown in Fig. 3. The degree of n-3 fatty acids incorporation was increased by increasing the amount of lipase in the mixture, but a significant increase was not observed when the lipase was present at a level greater than 10 wt.% of the total substrates. Thus, 10 wt.% loading of the total substrates was sufficient to saturate the reaction system in terms of lipase loading for the rest of experiments.

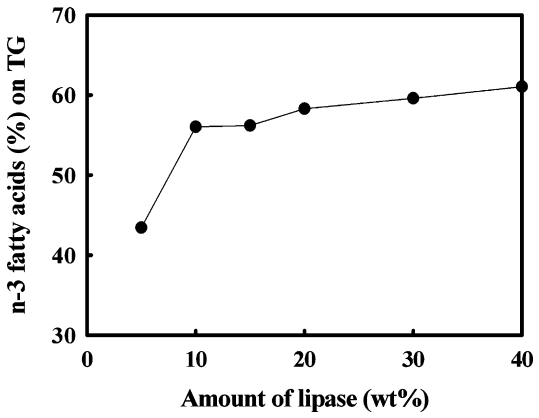

Fig. 3. Effects of amount of lipase on total n-3 fatty acids on TG under 10.2 MPa and 323.15 K $SCCO_2$, enriched n-6 TG/n-3 PUFA = 1/4, and co-solvent loadings 10 wt.% of total substrates.

3.2. Reaction time

It is important to determine the proper reaction time in order to reach an economic operating cost. Fig. 4 shows the total n-6 and n-3 contents on TG during the reaction time course with the enriched n-6 TG/n-3 PUFA = 1/4 and enzyme loading 10 wt.% and co-solvent content at 10.2 MPa and 323.15 K $SCCO_2$. The total n-3 PUFA content on TG increases with time up to 6 h, beyond which it remains relatively constant at 56%. Normally, the resistance of adsorption and de-adsorption between enzyme and substrates essentially determines the reaction time. As mentioned above, due to its high diffusivity and low surface tension, $SCCO_2$ can enhance the mass transfer rate, which leads to the fast formation of the enzyme–substrate complex at the initial reaction step. Likewise, the n-6 PUFA content on TG decreased to 13.1% after 6 h. After considering the cost, 6 h was chosen as the reaction time in this study.

3.3. Pressure effect

Fig. 5 shows the time course of the ratio of n-6/n-3 on TG of borage oil under $SCCO_2$ in the range of 10.2–20.4 MPa and 323.15 K. It demonstrates that the ratio quickly reaches 4 as

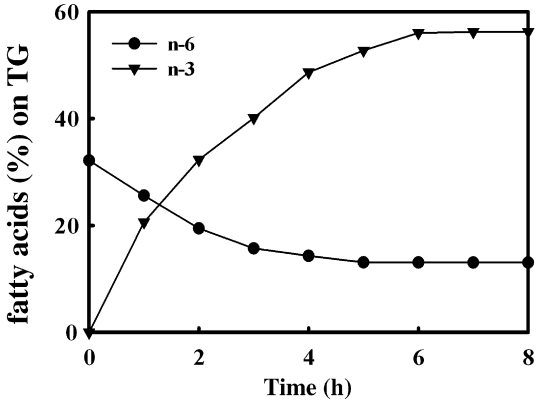

Fig. 4. The time course of total n-6 and n-3 contents on TG at 10.2 MPa and 323.15 K $SCCO_2$ with enriched n-6 TG/n-3 PUFA = 1/4 and enzyme and co-solvent loadings 10 wt.% of total substrates, respectively.

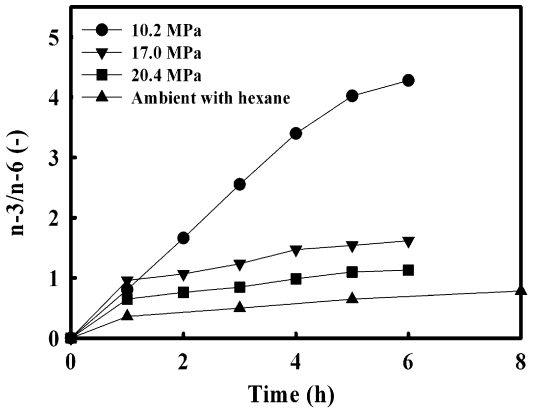

Fig. 5. The time course of the ratio of total n-3 to n-6 on TG for various pressures and 323.15 K $SCCO_2$, enriched n-6 TG/n-3 PUFA = 1/4, and enzyme and co-solvent loadings 10 wt.% of total substrates, respectively.

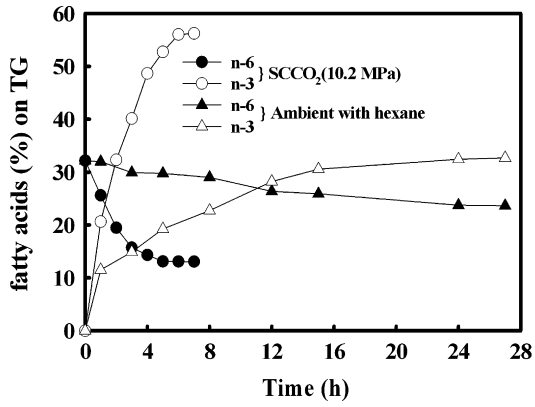

Fig. 6. Comparison of the time course of n-3 PUFA on TG under 10.2 MPa $SCCO_2$ with hexane solvent at ambient.

system pressure operates at 10.2 MPa after 6 h. Erickson et al. [30] reported that the activity of the enzyme can remain constant or even be elevated due to the aggregation of substrates around the enzyme in $SCCO_2$. This aggregation of substrates is caused by the enhanced clustering ability of CO_2 at supercritical conditions. This cluster of $SCCO_2$ additionally promotes the solubility of substrate and results in an increase of the substrate concentration around the enzyme. Besides, Ikushima et al. [31] have reported that $SCCO_2$ can promote the activation of the enzyme and provokes drastic conformational changes of the enzyme, causing the movement of the surface groups and creating an active site producing stereoselective machinery. Furthermore, accounting for the low viscosity and high diffusivity of $SCCO_2$, the substrates are easier to be transferred into pores of the immobilized carrier to contact with enzyme. The ratio becomes decreasing while system pressures are elevated over 10.2 MPa. The reason could be due to increasing activation energy of the esterification reaction at higher system pressures. Hence, 10.2 MPa was selected as the optimal operating pressure for further study.

Fig. 5 also shows that the ratio of n-3/n-6 content on TG at $SCCO_2$ is noticeably higher than that with hexane as a solvent at ambient. Previously, hexane was found to be the best solvent for enzymatic incorporation of DHA into borage oil at ambient condition because it can preserve enzyme activity via its hydrophobic property to maintain a layer of essential water around the enzyme molecules [16,28]. However, compared with the n-3 incorporation on TG in hexane solvent, the incorporation under 10.2 MPa $SCCO_2$ was 2.5 times higher at 6 h, as shown in Fig. 6. Even when the reaction time was increased to 27 h, the ratio of n-3/n-6 on TG in hexane solvent only reached 1.4. Accordingly, it demonstrates that $SCCO_2$ is a superior solvent for this enzymatic esterification compared to organic solvents.

3.4. Temperature effect

Based on reaction kinetics, the reaction rate becomes faster at higher temperatures. Fig. 7 demonstrates an increasing trend of the ratio of n-3/n-6 on TG with increasing temperature. From this figure, the best temperature for the higher ratio was 333.15 K at first 4 h, after which 323.15 K became having the highest ratio with close to 4. It is recognized that denaturation occurs in most proteins at 318–323 K and becomes nastier beyond 328 K. By raising the system temperature, enzyme would increase the kinetic energy and tendency of motion, and leads to a higher collision frequency with the surrounding molecules. When reaching a higher temperature, the enzyme may become denatured. Meanwhile, Kamat et al. [32] reported that below 313.15 K $SCCO_2$ can easily react with the free amino group on the surface of enzyme to form carbamate–enzyme complex via a covalent bonding. This carbamate–enzyme complex will reduce the activity of the enzyme, but this can be avoided by elevating the system temperature. Hence, by considering the stability and activity of enzyme, and the energy saving and cost, 323.15 K would be used as the optimal reaction temperature.

3.5. The effect of substrate molar ratio

Fig. 8 shows the effects of the substrate molar ratios on the ratio of n-3/n-6 on TG under 10.2 MPa and 323.15 K $SCCO_2$ for 6 h. At a fixed enriched n-6 TG concentration, the ratio of n-3/n-6 on TG is increased by decreasing the substrate molar ratios by raising the concentration of free n-3 PUFA, as seen in Fig. 8(a). When substrate molar ratio was up to 1/4, the ratio

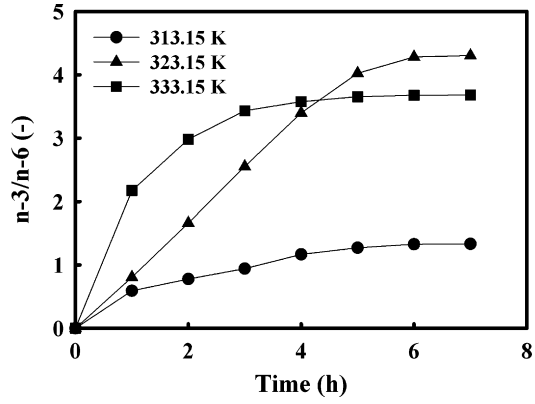

Fig. 7. The time course of the ratio of total n-3 and n-6 on TG for various temperatures and 10.2 MPa $SCCO_2$, enriched n-6 TG/n-3 PUFA = 1/4, and enzyme and co-solvent loadings 10 wt.% of total substrates, respectively.

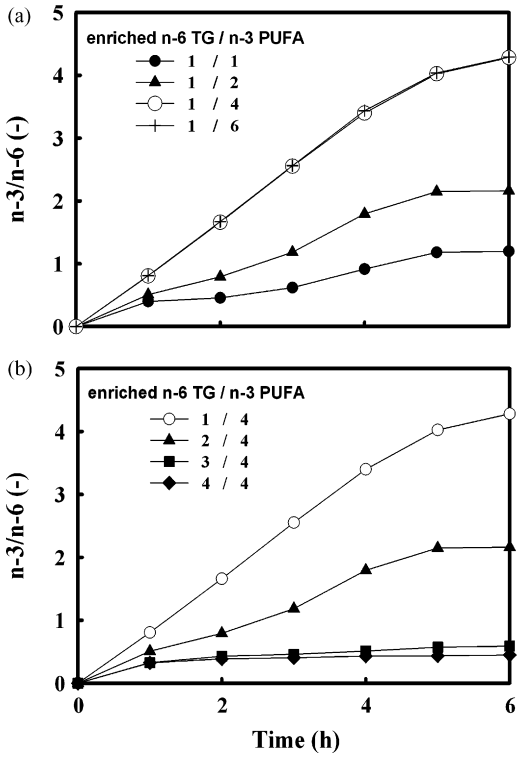

Fig. 8. Effects of substrate molar ratio on the time courses of the ratio of n-3 to n-6 on TG under 10.2 MPa and 323.15 K $SCCO_2$ for 6 h, and enzyme and co-solvent loadings 10 wt.% of total substrates, respectively. Reaction procedure: in case (a) decreasing the molar ratio by raising the concentration of free n-3 PUFA at a fixed enriched n-6 TG, and case (b) increasing the molar ratio by raising the concentration of enriched n-6 TG at a fixed free n-3 PUFA.

of n-3 to n-6 on TG reached around 4 after 6 h. After further decreasing the substrate molar ratio to 1/6, no significant change was observed. By considering the cost, 1/4 of substrate molar ratio was recommended. Meanwhile, at a fixed concentration of free n-3 PUFA, the increase of the substrate molar ratio by increasing the concentration of enriched n-6 TG was found to have a significantly negative effect on the total content of n-3, as shown in Fig. 8(b). It might be due to that the activity of enzyme could be inhibited by higher concentration of the enriched n-6 TG substrate. Because the viscosity of enriched n-6 TG is higher than that of n-3 PUFA, a layer of the excessive enriched n-6 TG might be formed on the surface of enzyme to prohibit contact between substrate and active site of enzyme, and hence inhibit the trans-esterification. Another reason might be due to the stereo effect of the enriched n-6 TG. Since the enriched n-6 TG is a large molecule, the TG–enzyme complex hinders the other free n-3 PUFA to form a covalent bond with enzyme. This hindering mechanism is the so-called substrate inhibition in the kinetics of enzyme.

3.6. Co-solvent effect

In order to consider the safety, cost and toxicity, ethanol was employed as a co-solvent in this study to determine the optimal additive ratio under 10.2 MPa and 323.15 K $SCCO_2$ for 6 h, as shown in Fig. 9. From this figure, the total content of n-3 on TG was notably increased by 30 wt.% (from 22 wt.% to 52 wt.%) by adding ethanol from zero to 10 wt.% of the total substrates. This result may be due to a positive improvement on the solubility of substrates by adding a specific amount of organic co-solvents into the reaction. The increased solubility of substrates can augment the local density around the enzyme to intensify the trans-esterification. Further increasing the addition of ethanol to 15 wt.% has a negative effect on the conversion of n-3 on TG. Because ethanol itself has a smaller molecular weight and higher number density at higher percentage input, it makes ethanol is easier to occupy the enzyme active sites than substrates. This negative effect could be in consequence to the competition between ethanol and substrates for the enzyme active sites. Therefore, the optimal input of ethanol as co-solvent is suggested for 10 wt.% of the total substrates.

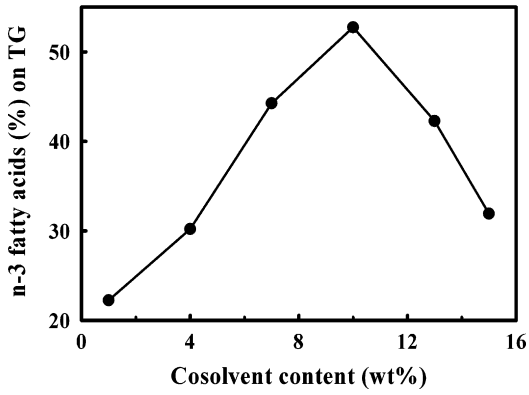

Fig. 9. Effects of amount of co-solvent content on total n-3 PUFA content on TG under 10.2 MPa and 323.15 K $SCCO_2$ for 6 h, enriched n-6 TG/n-3 PUFA = 1/4, and enzyme loadings 10 wt.% of total substrates, respectively.

3.7. Enzymatic stability

Fig. 10 shows the residual activity of IM60 changes in a semi-continuous operation under 10.2 MPa and 323.15 K $SCCO_2$ for 6 h per run. After seven pressurization and depressurization manipulations, the residual activity of the tested enzyme

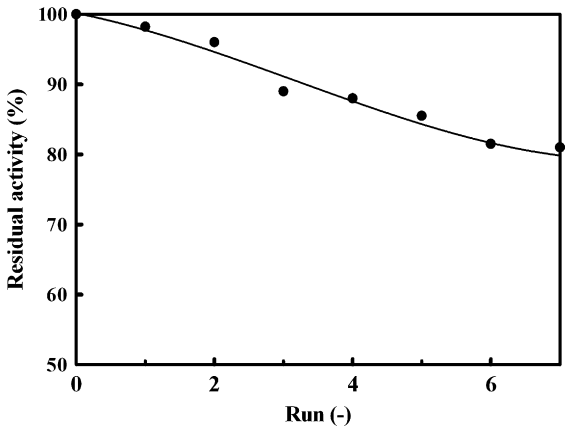

Fig. 10. Residual activity of IM60 changes with a semi-continuous operation for seven runs under 10.2 MPa and 323.15 K $SCCO_2$ for 6 h, enriched n-6 TG/n-3 PUFA = 1/4, and enzyme and co-solvent loadings 10 wt.% of total substrates, respectively.

remains 81% of the initial activity and the total content of n-3 on TG retained around 56 wt.%. The cause of the reduction of activity can be attributed to two key reasons. First, during the depressurized manipulation, the surface of water molecules may be vaporized, which may lead to a minor conformation change of the enzyme and reduce the activity. Second, the extended duration of the enzyme in a high temperature environment may cause the decay of activity. Although the residual activity has some reduction, the IM60 is rather stable and suitable in $SCCO_2$ with only 2.0% decay per experiment.

3.8. Investigation of reaction kinetics

Because the selected reactive substrates were not pure in this study, the following three experimental assumptions were made in order to reduce the reactive parameters:

(1) Because total n-3 PUFA content from urea inclusion method is up to 80.3%, all free fatty acids could be set to the same reactive substrate (n-3 PUFA).
(2) Because the mono-TG content from a lipase-catalyzed selective partial hydrolysis is up to 80.5%, all glycerides species in the reaction may be lumped together and be treated as the same reactive substrate (mono-TG).
(3) The formation of the ester bond was the rate-determining step.

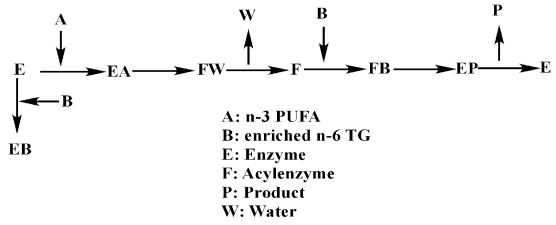

Fig. 11. Schematic representation of the ping-pong with single substrate inhibition by enriched n-6 TG mechanism.

Therefore, this lipase-catalyzed esterification was simplified to a reaction of di-substrates and di-products. According to Dumont et al. [33], the active sites on lipase execute the process of acylation through a charge relay system, which is similar to the ping-pong mechanism. Besides, from the above results, it is known that enriched n-6 TG has the effect of substrate inhibition on the reaction. Hence, the ping-pong mechanism with single substrate inhibition, similar to the equation proposed by Segel [34], was employed to describe this esterification, as shown in Fig. 11. From this figure, n-3 PUFA (A) was lined to the enzyme (E), leading to a substrate–enzyme complex, EA. After the formation of EA, the recombination of intra-molecule (FW) occurred, and an intermediary acylenzyme (F) was produced by delivering water (W) out. This was followed by the linkage of enriched n-6 TG (B) into a second complex that delivered

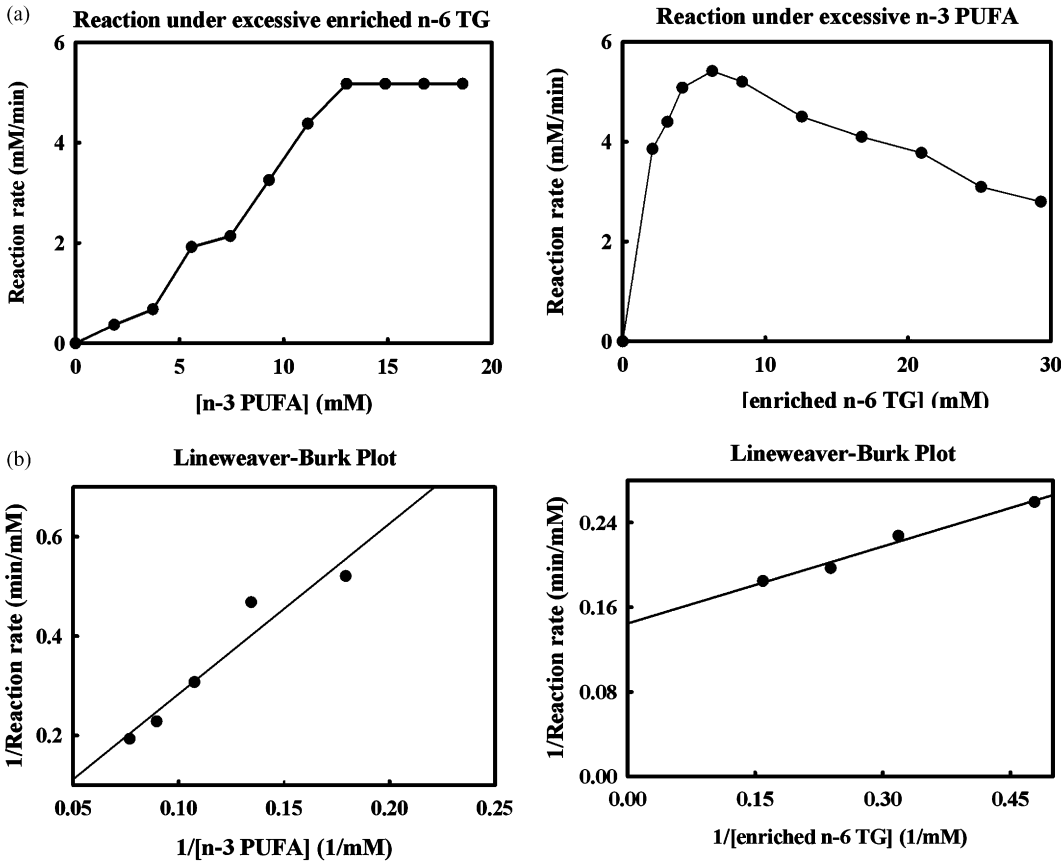

Fig. 12. Effects of one single substrate concentration on initial reaction rate (30 min) by keeping the other substrate oversaturated with Lineweaver–Burk plot in (a) excess n-3 PUFA and (b) excess enriched n-6 TG under 10.2 MPa and 323.15 K $SCCO_2$, enzyme and co-solvent content 10 wt.% of total substrates, respectively.

Table 4
Evaluating the reaction constants by Lineweaver–Burk plot

K_m^A (mM)	K_m^B (mM)	K_i^B (mM)	V_m (mM/min)
14.95	1.68	18.46	6.92

the product (P) and ensured enzyme recovery. The inhibition occurred as enriched n-6 TG (B) was lined to the enzyme (E), leading to a dead-end complex (EB).

Since the amount of product *A* is small at the beginning reaction, the concentration of *A* can be neglected. Based on the above assumptions and the proposed reaction mechanism, the initial equation of reaction rate (*V*) can be derived for this di-substrates enzyme-catalyzed reaction:

$$V = \frac{V_{max}[A_0][B_0]}{K_m^A[B_0](1 + [B_0]/K_i^B) + K_m^B[A_0] + [A_0][B_0]} \quad (1)$$

where $[A_0]$ and $[B_0]$, respectively represent the initial molar concentration of n-3 PUFA and enriched n-6 TG; K_m^A and K_m^B are their affinity constants; K_i^B is the inhibition constant of enriched n-6 TG; V_{max} is the initial maximum reaction rate. Eq. (1) can be further reduced as a modified Michaelis–Menten equation (e.g., $V = V_{max}[B_0]/[B_0] + K_m^B$ as $[A_0]$ is oversaturated) by keeping one substrate concentration oversaturated to a pseudo one substrate system. With the aid of the Lineweaver–Burk double-reciprocal plot, the kinetic constants, namely K_m^A, K_m^B, K_i^B and V_{max} in the modified Michaelis–Menten equation can be evaluated.

Fig. 12 shows two examples of the effects of one single substrate concentration on initial reaction rate by keeping an excessive substrate of the enriched n-6 TG or n-3 PUFA with Lineweaver–Burk plots. For the case of the excessive enriched n-6 TG, the reaction rate increases linearly (noted as a first-order reaction) with the concentration of n-3 PUFA up to 13 mM, after which the reaction rate becomes almost unchanged (noted as a zero-order reaction), as shown in Fig. 12(a). For the second case of the excessive n-3 PUFA, the reaction rate increases sharply to a maximum at 6.28 mM enriched n-6 TG, and then decreases significantly while further adding the enriched n-6 TG, as shown in Fig. 12(b). This result indicates that the higher enriched n-6 TG has a negative effect on the reaction rate, which is matched with the previous mentioned substrate inhibition.

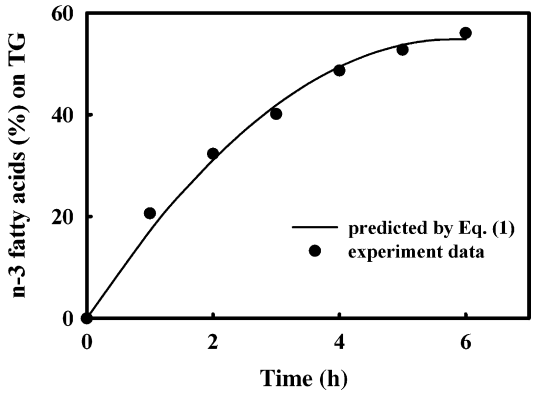

Fig. 13. Comparing the time course of total n-3 PUFA on TG between the predicted results by Eq. (1) and experimental data under 10.2 MPa and 323.15 K $SCCO_2$, enriched n-6 TG/n-3 PUFA = 1/4, and enzyme and co-solvent loadings 10wt.% of total substrates, respectively.

Following the above method, the values of the kinetic constants for this lipase-catalyzed trans-esterification reaction can be obtained and were shown in Table 4. Comparing values of the apparent affinity constants of K_m^A and K_m^B, it is noted that the affinity of enriched n-6 TG reacting to acylenzyme is larger than that of n-3 PUFA reacting to enzyme. In addition, K_m^A and K_i^B are roughly the same, which means that both n-3 PUFA and enriched n-6 TG have a competitive inhibition for the active sites on enzyme. It also indicates that the influence of inhibition becomes more serious if the enriched n-6TG has higher concentration. In order to verify this kinetic model, a typical experiment with operation conditions under 10.2 MPa and 323.15 K $SCCO_2$, enriched n-6 TG/n-3 PUFA = 1/4, and enzyme and co-solvent loadings 10 wt.% of total substrates, is carried out to compare with the predicted data by Eq. (1), as shown in Fig. 13. It demonstrates the predicted content of the total n-3 PUFA is matched fairly well to the experimental data with a deviation of only 2.1%.

4. Concluding remarks

The synthesis structured lipid with n-3/n-6 = 4 by lipase-catalyzed trans-esterification reaction under supercritical conditions has been investigated in this study. The reaction seems like a zero-order reaction up to 6 h, and then does not proceed any more. At 323.15 K, the enzyme has the best thermal stability. With pressure at 10.2 MPa, the enzyme has the best conversion efficiency. Due to the limitation of the substrate, the optimum molar ratio of enriched n-6 TG to n-3 PUFA is 1/4. The optimal inputs of enzyme and ethanol as co-solvent are 10 wt.% of the total substrates. The reaction operating under 10.2 MPa and 323.15 K $SCCO_2$ can obtain 56% of n-3 on TG and n-3/n-6 = 4 at 6 h, which is superior to that using hexane at ambient only reaching to 18% of n-3 on TG and n-3/n-6 = 0.7, respectively. From the results, $SCCO_2$ demonstrates a better catalytic ability and stability with respect to conventional hexane solvent, including high diffusivities and low viscosities, which increase mass transfer of substrate into the catalyst particles. The activity of the enzyme maintains 81% of initial activity after seven times of pressurization/depressurization. The reaction can be modeled by a modified Michaelis–Menten equation, and with the aid of the Lineweaver–Burk plot the kinetic constants were evaluated as $K_m^A = 14.95$ mM, $K_m^B = 1.68$ mM, $K_i^B = 18.46$ mM, and $V_{max} = 6.92$ mM/min. The predicted result of n-3 incorporating on TG is matched quite well to the experimental data with a deviation of only 2.1%.

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