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Characteristics of esterified rice bran oil converted by enzymatic esterification

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Abstract

In the present study, esterified rice bran oil (ERBO) was characterized using enzymatic esterification to improve stability, prevent acidification, enhance health-promoting biological activity and generate ω-3 PUFA-rich rice bran oil (RBO). Esterification reactions using RBO and ethanol were performed at 50°C under 200 bar with 3% lipozyme TL-IM (*Thermomuces lanuginosa* immobilized on silica gel) or RM-IM (*Rhizomucor miehei* immobilized on ion exchange resin) for 3 hr under supercritical CO_2. The molar ratios of ethanol to RBO were 3, 6, 9 and 12, respectively. Total lipid contents and acid values decreased (maximum 83.75%),but γ-oryzanol content increased (maximum 41.33%) in esterified RBO (ERBO) prepared using TL-IM or RM-IM. In addition, DPPH radical scavenging activity of ERBO prepared by RM-IM at an ethanol to RBO molar ratio of 3 was 0.02 μg μl^{-1}, which was 63-fold higher than that of α-tocopherol (IC_{50}=1.25 μg μl^{-1}). The anti-inflammatory effect of RM-IM 1:3 hydrolysate of RBO was verified showing its suppressive effect towards iNOS and Cox-2mRNA expression in a dose-dependent manner. Therefore, ERBO is a promising source of functional food, cosmetics and pharmaceuticals.

Key words

Lipase, Rice bran oil, Supercritical carbon dioxide, Transesterification

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Introduction

Transesterification can be used to enhance the value of oils. Low-value oils and fats can be converted to bio-diesel, trans-free fats, medium chain-length triglycerides and ω-3 polyunsaturated fatty acid (PUFA)-rich plant oils using several methods such as methanolysis, interesterification and acidolysis. Transesterification is a reversible reaction accelerated by the presence of a catalyst. During conventional chemical processing, synthesis of esters by transesterification is achieved by either acid or alkaline esterification. This can lead to reduced selectivity and undesirable side reactions (Soumanou and Bornscheuer, 2003; Meher *et al*., 2006). Lipase catalysts can be used to modify the properties of esterified lipids by altering the locations of fatty acid in glyceride and replacing one or more fatty acids with new fatty acids. These exchange reactions usually proceed with high regio- and/or enantio-selectivity, rendering lipases an important group of biocatalysts (Jaeger and Reetz, 1998). Using this approach, a relatively inexpensive and less desirable lipid can be modified into a more valuable lipid (Sharma *et al*., 2001; Oliveira and Oliveira, 2001).

Nutraceuticals and controlled-release medicinal tablets are generated using food grade monoglycerides. Diglycerides have recently attracted attention for preventing obesity and for their anti-diabetic and anti-atherosclerotic properties (Moquin *et al*., 2006). R1, R2 and R3 are fatty acids acylated to SN_1-, SN_2-, and SN_3-positions, respectively, according to the stereochemical nomenclature. Some lipases are specific for promoting esterification in SN_1 and SN_3 positions, leaving the fatty acid moiety at SN_2 position intact. Rice bran oil (RBO) contains high percentage of monoglyceride PUFAs at SN_2 position. Supercritical fluid (SCF) technology is a rapidly growing alternative to more conventional methods of extraction, reaction, fractionation and analysis. In supercritical state,densities are liquid-like, and viscosity is similar to that of normal gases, with

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diffusivities approximately two orders of magnitude higher than those of typical liquids (Brunner, 2005). In addition, the solubility of solutes in SCFs is strongly influenced by density of supercritical fluid which can be easily and rapidly controlled by mild variation in temperature and pressure (Jaeger and Reetz, 1998). Lipases are well-suited for supercritical CO_2(SC-CO_2) applications, because their catalytic effect involves a lipid-water interface. These characteristics provide an inherent affinity for hydrophobic media, and it is well known that the catalytic efficiency of enzymes decreases as hydrophilicity of the reaction medium increases (Laudani *et al*., 2007).

The objective of the present study was to characterize esterified rice bran oil (ERBO), obtained by ethanolysis, to investigate the efficiency of production of ω-3 PUFA-rich diglycerides, monoglycerides and ethyl esters, and to measure acid values, anti-oxidative activities and anti-inflammatory activities of ERBO, respectively.

Materials and Methods

Rice samples (*Oryza sativa* LINN. var. *japonica*; the Korean cultivars, Dongjin; Gramineae) used in the present study were harvested in Gijang, Busan during the fall of 2011, and rice bran was milled and provided by PN RICE Co., Ltd. (Kimhae, Gyeonsanggnamdo, Korea) in March 2012. Supercritical CO_2 extraction from 57 kg rice bran powder was performed at 32 ºC and 270 bar for 2 hr in a semi-continuous flow-type apparatus with a 3-l extractor, as described by Choi *et al*. (2014). The resulting RBO was 9.5 kg, and its mean molecular weight was 860 g mol^{-1}. Immobilized commercial lipases of specific lipozymes TL-IM (*Thermomuces lanuginosa* immobilized on silica gel) and RM-IM (*Rhizomucor miehei* immobilized on ion exchange resin) were purchased from Novozymes (Bagsvaerd, Denmark). According to the specifications, specific activities of lipozyme TL-IM and RM-IM were 250 and 150 IUN g^{-1}, respectively. The diameters of immobilized enzyme particles ranged from 0.2 to 1.0 mm. The solvents, including ethanol for transesterification, and other reagents used were of analytical grade. All the solutions were made with ultrapure deionized water (Milli-Q advantage A10 ultrapure water purification system; Millipore, Billerica, MA, USA).

In a conventional solvent-free system, hydrolysis rate was evaluated by measuring the acid value for comparison with ethanolysis rate. The experiment was performed in 25 ml Erlenmeyer flasks containing mixture of 5 g RBO and 0.103 ml ultrapure water [Rw (molar ratio of water to RBO) = 1.0] with TL-IM and RM-IM lipozymes (0.5-5.0 wt% load to RBO). The reaction mixture was incubated at 50 ºC and agitated at 120 rpm in a shaking incubator (VS-8480SRN-L, Vision Scientific Co. Ltd, Deajeon, Korea). The reaction yield of ethyl ester was calculated as equivalent to theoretical value / experimental value ×100.

Following the reaction, acid value of all the samples were determined according to the International Standard ISO 660:2009 (2009). Briefly, the sample was dissolved in a mixture of ethanol and diethylether and titrated with potassium hydroxide solution to phenolphthalein end-point. Free fatty acid molar amount was calculated by the following equation:

$$n[FA] = A.V. \times m[oil] / M[KOH],$$

where n[FA] is mole of free fatty acid (mol); A.V. is acid value (mg g^{-1}); m[oil] is mass of oil (g) and M[KOH] is molar mass of KOH (mg mol^{-1}).

Ethanolysis in a supercritical CO_2(SC-CO_2) system : Enzymatic ethanolysis in the SC-CO_2 system was performed in a cylindrical high pressure stainless steel (SS-316) vessel with an internal volume of 1,100 ml (289 mm × 70 mm I.D.), which consisted of magnetic driver and water jacket. Reactor temperatures were measured with an overall accuracy estimated within ± 0.2 K. Pressure was set at 200 bar. In the semi-batch type supercritical fluid mode, ethanol was pipetted directly into the reactor along with enzyme and RBO. After injection, the reactor was closed, and pressure was increased. In this mode, pressure was kept constant without any net flow of carbon dioxide through the reactor (static period), and after a predetermined time the products were extracted dynamically using metering valve. Finally, the reactor was depressurized to atmospheric conditions, and hexane was added to the residue for washing.

Gas chromatography analysis : Fatty acid methyl ester mixture (FAME) was prepared by esterification using alcoholic sulfuric acid reagent according to the International Union of Pure and Applied Chemistry procedure (IUPAC, 1987). The methyl ester was analyzed using Hewlett-Packard 5890 II GC (Hewlett-Packard Co., Palo Alto, CA, USA) equipped with a capillary column type DB-wax (i.d. 0.25 mm × 30 m, 0.25 μm, Agilent, Santa Clara, CA, USA). The injection volume of the diluted samples in hexane was 1 μl, and the split ratio was fixed at 100:1. The temperature program was as follows: 40 ºC for 5 min, 10ºC min^{-1} to 180ºC, 5ºC min^{-1} to 260ºC and 260ºC for 5 min. A FID detector was used at 250ºC. Hydrogen at a flow rate of 2 ml min^{-1} was used as carrier gas, and the inlet temperature was fixed at 250°C. It was assumed that the ratio of single component peak area to total peak area represent mass fraction of the component. Lipid standards (37 components of FAME mixture, Supelco, Bellefonte, PA, USA) were used for identification.

Analysis of Γ-oryzanol : Γ-oryzanol content was determined by spectrophotometer (UVIKON XS; SECOMAM, Alés, France) at 315 nm according to the method of Kim and Kim (1991). Briefly, the samples were diluted 1/200 using chloroform (Duksan Co. Ltd., Ansan, Korea), and A_{315} of each sample was determined. Γ-oryzanol content was calculated by γ-oryzanol standard curve. Authentic γ-oryzanol used in the study was purchased from Oryza Oil& Fat Chemical Co. Ltd. (Tokyo, Japan).

Scavenging effects of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals : DPPH radical scavenging activity was determined

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according to the method of Blois (1958) with minor modifications. Briefly, the sample dissolved in 50 µl ethanol was added to 0.15 mM DPPH radical solution (200 µl). The mixture was kept in dark for 30 min at 37°C, and absorbance was read at 520 nm by an ELISA reader (Spectra MAX 190; Molecular Devices, Sunnyvale, CA, USA). DPPH radical-scavenging rate (%) was calculated using the following equation:

$$\text{EDA (electron donating ability)} = [1 - (A - C/B)] \times 100$$

where A is absorbance of the sample when a blank was substituted for ethanol; B is absorbance of the sample when a color-contrast agent was substituted for ethanol in DPPH radical–ethanol solution, and C is absorbance of the color-contrast agent alone. Tocopherol (CAS No. 10101-41-0, Junsei Chemical Co., Ltd., Tokyo, Japan) was used as positive control.

Cytotoxicity assay : RAW264.7 cells (American Type Culture Collection, Rockville, MD, USA) were grown in Nunc flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 10 mM HEPES, 2 mM L-glutamine, 0.2% $NaHCO_3$, 1 mM sodium pyruvate and 10% (v/v) heat-inactivated fetal bovine serum (FBS) in a humidified chamber (Forma 3111, Thermo, Germany) with 5% CO_2 / 95% air at 37°C.

Cytotoxicity was determined as described by Choi *et al*. (2013). Cellular toxicity of various concentrations of RM-IM 1:3 of RB-SCE was assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, which was based on the conversion of MTS to formazan by mitochondrial dehydrogenases. The formazan generated was measured at 492 nm by Spectra Max 250 ELISA Reader (Molecular Devices, USA). A commercial MTS assay kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay), and MTS were purchased from Promega (Madison, WI, USA). Cell viability was expressed as percentage of control value.

RNA extraction and RT-PCR : RAW264.7 cells cultured using the above methods were seeded in six-well culture plates at a density of 2 × 10^5/well and incubated for at least 24 hr to allow adherence to the plates at 37 ºC in 5% CO_2 incubator (Forma 3111, Thermo, Germany). After washing three times with medium, ERBO-RM (1:3)(0.01 and 0.1 µl $µl^{-1}$)was added, and cells were cultured for the indicated time, after which the culture supernatants were collected. Total RNA was extracted using Total RNA Isolation Kit (MACHEREY-NAGELGmbH & Co. KG, Düren, Germany), and cDNA was synthesized by reverse transcription using ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) in a 20 µl mixture containing 1 µg RNA, 50 mM KCl, 10 mM Tris/HCl, 5 mM $MgCl_2$, 1 mM each dNTP, oligo-(dT) primers, 20 U RNAse inhibitor and 50 U MuLV reverse transcriptase. Nucleotide sequences of the primers for inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (Cox-2) and β-actin are provided in Table 1. The reaction mixture was incubated for 5 min at 94ºC and then heated for 45 sec at 94°C, 45 sec at 50°C and 2 min at 72ºC for iNOS in thermocycler (PTC-1148, Bio-RadLaboratories, Inc., Hercules, CA, USA). For COX-2 and β-actin, the reaction mixture was incubated for 2 min at 94ºC and then heated for 30 sec at 94ºC, 30 sec at 58ºC and 30 sec at 72ºCin a thermocycler for 40 cycles. Electrophoresis (Mupid®-2plus; ADVANCE Co., Ltd., Tokyo, Japan) was performed in 2% agarose gel (Agarose LE, Georgiachem) at 100 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid and 1 mM EDTA) for 50 min. Retardation of DNA was visualized on a UVGel document system (I-MAX-MC2000, CoreBio, Ann Arbor, MI, USA). Quantification of iNOS and Cox-2mRNA expression was normalized to β-actin. The expression ratio (%) of each mRNA was calculated from the area ratio of each band using ImageJ (Image Processing and Analysis in Java, National Institutes of Health, Bethesda, MD, USA).

Table 1 : Nucleotide sequences of the primers used for PCR amplification

		Primer sequences
iNOS	Sense	5'-CCCTTCCGAAGTTTCTGGCACCACC-3'
	Antisense	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
COX-2	Sense	5'-CTGACCCACTTCAAGGGAGTCTGG-3'
	Antisense	5'-CCATCCTGGAAAAGGCGCAGTT-3'
β-actin	Sense	5'-TGACCGAGCGTGGCTACAGC-3'
	Antisense	5'-ACCGCTCATTGCCGATAGTG-3'

iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2

Results and Discussion

Esterification reactions using RBO and ethanol were performed at 50°C under 200 bar with 3% immobilized lipases (Lipozyme TL-IM and Lipozyme RM-IM) for 3 hr under supercritical CO_2. The molar ratios of ethanol to RBO were 3, 6, 9 and 12. The reaction yield of ethyl esters (%), prepared with ethanol to RBO molar ratios of 3, 6, 9 and 12 using 3% RM-IM, were 49.8, 74.6, 76.3 and 41.2%, respectively. The reaction yields (%) of ethyl esters using 3% TL-IM were 43.9, 68.8, 75.6 and 40.7%, respectively (Table 2). When the molar ratio of ethanol to RBO was increased from 3 to 9, the reaction yield of ethyl esters increased in both TL-IM- and RM-IM-treated groups. However, when molar ratio of ethanol to RBO was 12, the reaction yield of ethyl esters decreased significantly.

Acid values of ethyl esters, prepared at ethanol to RBO molar ratios of 3, 6, 9 and 12 using 3% RM-IM, were 46.07, 59.90, 39.76and 26.17. Acid value of ethyl esters using 3% TL-IM were 102.29, 96.57, 110.57and 93.06, respectively (Fig. 1a). Acid value of RBO was 161.06. Overall, the acid value of ethyl esters prepared using both TL-IM and RM-IM was lower than that of RBO. Acid value of ethyl esters, prepared by usingTL-IM, was lower than that of ethyl esters prepared using RM-IM.

Table 2 : Reaction yield of ethyl esters of rice bran oil (RBO) via enzymatic hydrolysis reactions using TL-IM and RM-IM, respectively.The ethanol to RBO molar ratios were 3, 6, 9 and 12, respectively

RBO (g)	Ethanol (g)	Mole ratio	Glycerol (g)	Unreacted and remained ethanol (g)	Ethyl esters (g)		Reaction yield (%)
					Theoretical value	Experimental value	
<RM-IM>							
129	20.7	1:3	13.8	-	135.9	67.8	49.8
129	41.4	1:6	13.8	20.7	135.9	101.5	74.6
129	62.1	1:9	13.8	41.4	135.9	103.8	76.3
129	82.8	1:12	13.8	62.1	135.9	56.1	41.2
<TL-IM>							
129	20.7	1:3	13.8	-	135.9	59.7	43.9
129	41.4	1:6	13.8	20.7	135.9	93.5	68.8
129	62.1	1:9	13.8	41.4	135.9	102.8	75.6
129	82.8	1:12	13.8	62.1	135.9	55.4	40.7

Reaction yield of ethyl esters = (theoretical value / experimental value) ×100; RBO: rice bran oil; ERBO-RM: esterified rice bran oil by RM-IM (*Rhizomucor miehei* immobilized on ion exchange resin); ERBO-TM: esterified rice bran oil by TL-IM (*Thermomuces lanuginosa* immobilized on silica gel)

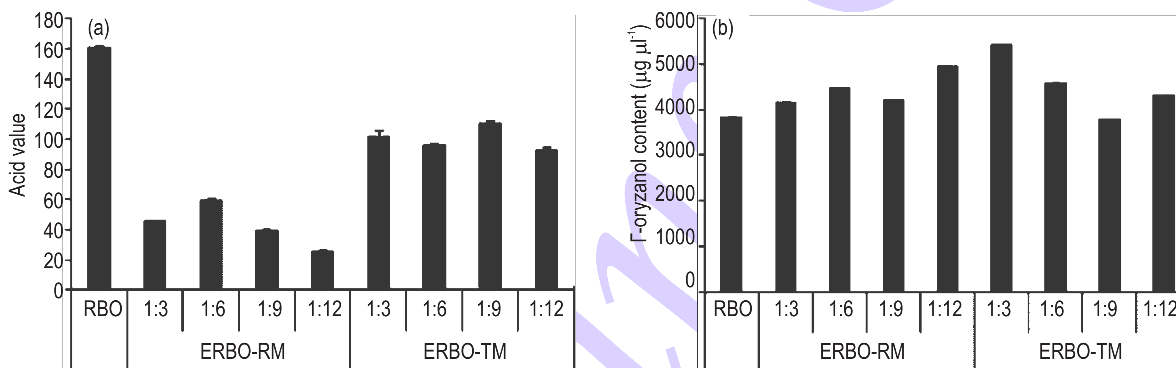

Fig. 1 : The acid values (a)and γ-oryzanol content (b)of ethyl esters prepared with ethanol to RBOmolar ratios of3, 6, 9 and 12 using 3% RM-IM and TM-IM, respectively; RBO: rice bran oil; ERBO-RM: esterified rice bran oil by RM-IM (*Rhizomucor miehei* immobilized on ion exchange resin); ERBO-TM: esterified rice bran oil by TL-IM (*Thermomuces lanuginosa* immobilized on silica gel)

The γ-oryzanol content of ethyl esters, prepared with ethanol to RBO molar ratios of 3, 6, 9 and 12 using 3% RM-IM, were 4172.00, 4478.67, 4218.67 and 4947.56 μg μl^{-1}. The γ-oryzanol content of ethyl esters, using 3% TL-IM, were 5443.11, 4596.44, 3798.67and 4329.78μg μl^{-1} (Fig. 1b). The γ-oryzanol content of RBO was 3851.33μg μl^{-1}. Overall, γ-oryzanol content of ethyl esters prepared using both TL-IM and RM-IM was higher than that of RBO. The γ-oryzanol content of ethyl esters, prepared by using 3 molar ratio of ethanol to RBO using TL-IM, was highest among all groups evaluated.

Change in the fatty acid content of RBO prepared by enzymatic esterification using RM-IM and TL-IM at different ethanol to RBO molar ratios, is shown in Table 3. Palmitic acid content, as a major component of RBO, did not change significantly in any of the groups evaluated. Oleic acid and linoleic acid contents, as other major components of RBO, decreased in all the test groups, excluding two groups treated with ethanol to RBO molar ratio of 12, using RM-IM and TL-IM.

The 50% inhibitory concentration (IC_{50}) values of DPPH radical scavenging effects of ethyl esters prepared with ethanol to RBO molar ratios of 3, 6, 9 and 12 using 3% RM-IM were 0.026, 0.033, 0.036 and 0.032 μg μl^{-1}. These values using 3% TM-IM were 0.037, 0.042, 0.033 and 0.040 μg μl^{-1}, respectively (Fig. 2). The IC_{50} value of DPPH radical scavenging effect of RBO was 0.035 μg μl^{-1}.

The IC_{50} value of DPPH radical scavenge activity of esterified RBO prepared using RM-IM at ethanol to RBO molar

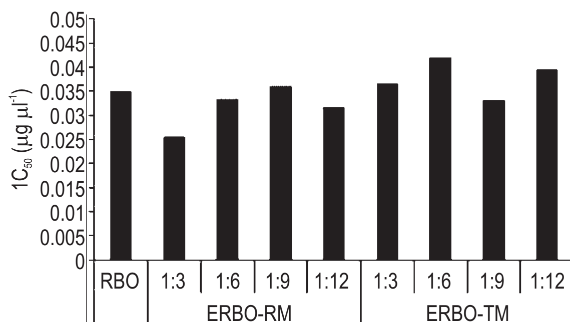

Fig. 2 : The IC$_{50}$ of DPPH radical scavenging activity of ERBO prepared by enzymatic esterification using RM-IM and TM-IM at ethanol to RBO different molar ratios. The molar ratios of ethanol to RBO were 3, 6, 9 and 12. The IC$_{50}$ value of DPPH radical scavenging effect of RBO was 0.035 µg µl^{-1}; IC$_{50}$: 50% inhibitory concentration; RBO: rice bran oil; ERBO-RM: esterified rice bran oil by RM-IM (*Rhizomucor miehei* immobilized on ion exchange resin); ERBO-TM: esterified rice bran oil by TL-IM (*Thermomuces lanuginosa* immobilized on silica gel)

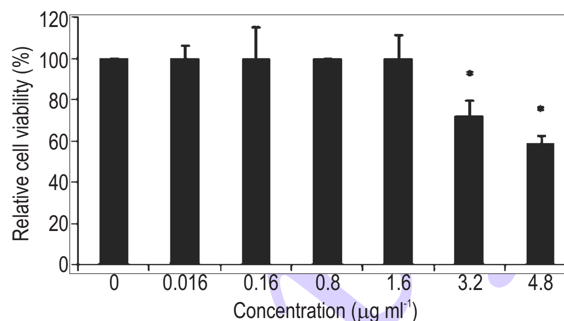

Fig. 3 : Effects of RM-IM 1:3 from RBO at concentrations of 0.16, 0.32, 0.8, 1.6, or 3.2 µg ml^{-1} on the viability of RAW 264.7 cells after 24 hr of treatment based on MTS assay; *Denotes a significant difference from controls at $P < 0.05$

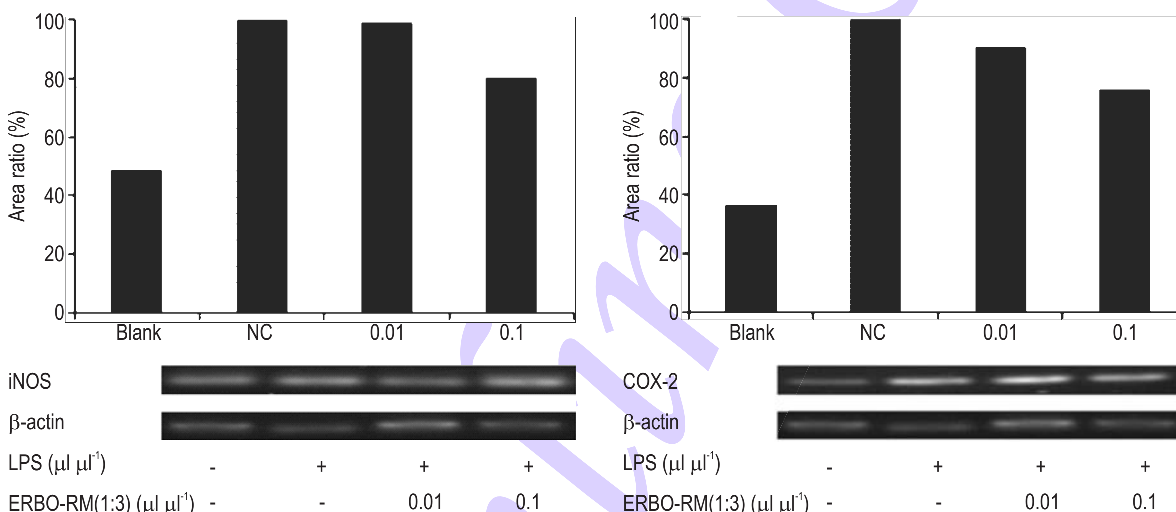

Fig. 4 : Anti-inflammatory effectof RM-IM 1:3 hydrolysate from RBO was examined by evaluating its suppressive effect on iNOSmRNA expression(a) and COX-2mRNA expression (b); iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; LPS: lipopolysaccharide; ERBO-RM: esterified rice bran oil by RM-IM (*Rhizomucor miehei* immobilized on ion exchange resin); -: without; +: with

ratio of 3, was 0.026 µg µl^{-1} (lowest value among all the groups evaluated) which was 63-fold higher than that of α-tocopherol (IC$_{50}$=1.25 µg µl^{-1}).Based on these results, ERBO selected was prepared by enzymatic esterification using RM-IM (ERBO-RM(1:3)) at ethanol to RBO molar ratio of 3 for further experiments. Short-term cytotoxicity of RM-IM 1:3 hydrolysate of RBO was examined using MTS assay. As shown in Fig. 4, no inhibitory effect on cell viability was observed after 24 hr of incubation, using either 0.016, 0.16, 0.8 or 1.6 µg ml^{-1} ERBO-RM (1:3) (Fig. 3).

The anti-inflammatory effect of the RM-IM 1:3 hydrolysate of RBO was examined based on its suppressive effect on iNOS and Cox-2mRNA expression. RAW 264.7 macrophage cells were treated with either blank, negative control (only treated lipopolysaccharide; LPS), or 0.01 or 0.1 µg µl^{-1} RM-IM 1:3 hydrolysate from RBO (with LPS), and the expression ratios of iNOSmRNA were 29, 61, 60 and 49%, respectively (Fig. 4a). When RAW 264.7 macrophage cells were treated with blank, negative control (only treated LPS), or 0.01 or 0.1 µg µl^{-1} of RM-IM 1:3 hydrolysate from RBO (with LPS), the expression ratios of

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COX-2mRNA were 29, 81, 73 and 61%, respectively (Fig. 4b). ERBO-RM (1:3) inhibited iNOS and COX-2 mRNA expression in a dose-dependent manner in LPS-stimulated RAW 264.7 cells.

Esterification reaction was performed under a supercritical CO_2 reaction field. To maximize fluid delivery rates involved in the reaction, density of supercritical carbon dioxide was set at 0.789g cm^{-3} under 200 bar at 50 ºC, which was same as ethanol phase density conditions (0.789g/cm^3) (Table 2). In general, extra-cellular microbial lipases were more thermo-stable than animal and plant lipases. In addition, immobilized enzymes showed decreased susceptibility to denaturation by heat (Akoh and Min, 2002). Optimal temperature for most immobilized lipases ranged from 30 to 62 ºC, but temperature higher than 50ºC decreased enzyme activity due to denaturation. In addition, most proteins were vulnerable to temperature above 55ºC, and low temperature was desirable in terms of cost (Shibamoto 1994; Laudani *et al*., 2007; Zhao *et al*., 2007). Thus, 50ºC was suitable temperature for conventional solvent-free, organic solvent and SC-CO_2 systems used in the present study.

The molar ratio of ethanol to the substrate RBO (1:3, 1:6, 1:9, and 1:12) and two species of immobilized enzymes as response factors, involved in the enzymatic reactions in the reaction field of supercritical CO_2, were considered in this study. Although the theoretical molar ratio of esterification reaction was 1:3 (substrate: ethanol), esterification reaction itself underwent a "ping-pong" mechanism. Thus, in esterification reaction, stepwise addition of reactants or more reactants than theoretical molar ratio was required. In the present study, during all esterification reactions using two types of immobilized enzymes, the reaction yield increased up to amolar ratio of 1:9 (Table 2). For two immobilized enzymes, highest reaction yield was observed at a molar ratio of 1:9. The reaction yield decreased up to a molar ratio of 1:12, which was probably due to an inverse reaction or inhibition of enzymatic activity by increased ethanol content.

The ratio of saturated fatty acid: monounsaturated fatty acid: polyunsaturated fatty acid in total triglycerides in RBO was 0.73:1:0.93. Major saturated fatty acid was palmitic acid (18.86%), and major unsaturated fatty acids were oleic acid (34.16%) and linoleic acid (35.30%) in RBO (Table 3). After the enzymatic reaction in supercritical CO_2 phase, the fatty acid content of RBO decreased under overall reaction conditions, except in two groups treated with ethanol to RBO molar ratio of 12, using RM-IM and TV-IM (Table 3). Since palmitic acid, oleic acid and linoleic acid are major component in rice oil, the contents of saturated fatty acid palmitic acid were stable. The contents of unsaturated fatty acids, oleic acid and linoleic acid decreased at an increased ethanol molar ratio. Prior to the enzymatic reaction in supercritical CO_2 phase, the composition of RBO was mainly triglycerides. The acyl groups of triglyceride were cleaved in the enzymatic reaction, after which free fatty acids liberated from triglyceride were combined with ethanol to produce ethyl ester. Typically, liberation of fatty acids present in SN_1or SN_3 position on the outer acyl group was more likely to occur than that of fatty acids in SN_2 position. The structural nature of unsaturated fatty acids was bent shape due to double bond. Thus, unsaturated fatty acids were located stochastically in SN_1 or SN_3 position, and not SN_2 position of triglyceride.

In the present study, acid values of ethyl esters prepared with TL-IM and RM-IM enzymes were lower than that of RBO (Fig. 1a). After the enzymatic reaction in super critical CO_2 phase, all fatty acid contents decreased (Table 3). This might be due to free fatty acids present in the substrate before reaction preferentially participated in esterification reaction. Thus, as the free acid content in ERBO decreased, the acid value of ERBO also decreased.

According to the results of Choi *et al*. (2014), RBO prepared in super critical CO_2 and one of the major components, γ-oryzanol have hair growth-promoting activity. Excluding ERBO-TM (1:9), γ-oryzanol content increased in all the groups evaluated (Fig. 1b). Γ-oryzanol is an ester compound combined with terpene alcohols and ferulic acid (Patel and Naik, 2004). During RBO esterification reaction, terpene alcohols and ferulic acid in RBO could combine and generate γ-oryzanol, which might have increase γ-oryzanol content in ERBO.

The DPPH radical scavenging effects of ERBO were superior to those of RBO (Fig. 2). This might be because during esterification reaction, the amount of ferulic acid (which has potent anti-oxidative effects (Graf *et al*., 1992) increased. The amount of γ-oryzanol generated by ferulic acid ester during esterification reaction also increased. In addition, during esterification, triglycerides decomposed and generated monoglycerides and diglycerides. The average molecular weight of triglycerides was 800-900; otherwise, the average molecular weights of diglycerides and monoglycerides were 500-600 and 200-300, respectively. Thus, as the molecular weight of substances decreased, the activation energy decrease and the moving rate of material increase. This resulted in improved reactivity.

Short-term cytotoxicity within 24 hr was evaluated using various concentrations of RBO. No cytotoxicity was observed at <1.6 μg ml^{-1} RBO (Fig. 3); however, slight morphological changes in RAW 264.7 cells were observed at > 3.2 μg ml^{-1} RBO (data not shown). In accordance with ISO 10993-5 (2009), materials with more than 30% cell death are considered to be cytotoxic. Generally, fatty acids showed low cytotoxicity. Indeed, fatty acids including caprylic acid, capric acid, lauric acid and myristic acid exhibited no or low cytotoxicity at 0.6-2.5 mg ml^{-1}, showing 81.1-109.7 % of L-929 mouse fibroblasts cell survival (Souza *et al.,* 2014). These fatty acids exhibited sever or slight cytotoxicity at 6.0-25.0 mg ml^{-1}, depending on the type of fatty acid. As RBO is a mixture of esterified fatty acids, it is not easy to make comparison

Table 3 : Fatty acid content of RBOprepared by enzymatic esterification using RM-IM and TL-IM at different ethanol to RBOmolar ratios

Fatty acid	RBO	RM-IM (RBO: ethanol)				TL-IM (RBO: ethanol)			
		1:3	1:6	1:9	1:12	1:3	1:6	1:9	1:12
Caprylic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lauric acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Myristic acid	0.22	0.11	0.12	0.12	0.13	0.14	0.12	0.12	0.11
Pentadecanoic acid	0.0	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Palmitic acid	13.86	9.32	9.31	9.05	9.25	8.46	8.99	9.23	8.85
Palmitoleic acid	0.14	0.07	0.07	0.07	0.08	0.08	0.07	0.07	0.06
Heptadecanoic acid	0.04	0.02	0.02	0.02	0.03	0.02	0.02	0.02	0.02
Stearic acid	1.06	0.70	0.64	0.64	0.68	0.66	0.65	0.64	0.66
Oleic acid	34.16	23.95	22.55	22.10	22.21	25.07	22.30	22.28	24.20
Linoleic acid	32.30	23.69	22.52	21.76	21.85	25.19	21.83	21.96	21.63
Arachidic acid	0.33	0.27	0.24	0.24	0.23	0.23	0.24	0.23	0.25
cis-11-Eicosenoic acid	0.39	0.30	0.26	0.26	0.26	0.30	0.27	0.26	0.27
Linolenic acid	1.19	0.75	0.73	0.69	0.70	0.81	0.69	0.71	0.68
Heneicosanoic acid	0.0	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.03
cis-11,14-eicosadienoic acid	0.11	0.02	0.02	0.02	0.01	0.01	0.02	0.01	0.01
Behenic acid	0.0	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.08
Erucic acid	0.0	0.02	0.01	0.01	0.01	0.02	0.02	0.01	0.02
cis-11,14,17-Eicosatrienoic acid	0.0	0.01	0.02	0.01	0.01	0.01	0.01	0.03	0.01
Arachidonic acid	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Lignoceric acid	0.24	0.12	0.10	0.10	0.10	0.10	0.10	0.10	0.11
Nervonic acid	0.0	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
cis-4,7,10,13,16,19-docosahexaenoic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total (g $100g^{-1}$ oil)	84.07	59.53	56.77	55.24	55.69	61.26	55.47	55.81	57.04

RBO: rice bran oil; ERBO-RM: esterified rice bran oil by RM-IM (*Rhizomucor miehei* immobilized on ion exchange resin); ERBO-TM: esterified rice bran oil by TL-IM (*Thermomuces lanuginosa* immobilized on silica gel)

between cytotoxicity of fatty acid and cytotoxicity of RBO. In the present study, no inhibitory effects on cell viability were observed after 24 hr of incubation using <1.6 μg ml^{-1} RBO (Fig. 3). The relative cell viability at>3.2 μg ml^{-1} showed 71.9%, which was considered to be cytotoxic. Therefore, cytotoxicity of RBO assumed similar level with those of other fatty acids.

ERBO-RM (1:3) inhibited iNOS and COX-2 mRNA expression in a dose-dependent manner in LPS-stimulated RAW 264.7 cells (Fig. 4a and b). This might be because during esterification reaction, the amount of ferulic acid (which is reported to have potent anti-inflammatory effects (Srinivasan *et al*., 2007) increased. In addition, as discussed above, the anti-inflammatory effects and biological activities improved due to lower average molecular weight of diglycerides and mono glycerides after esterification reaction.

In conclusion, ERBO possessing lower acid values and various beneficial health effects, including its anti-oxidant and anti-inflammatory activities, was prepared by enzymatic esterification in a super critical CO_2 system with out any disadvantage like discoloration or malodor. Therefore, ERBO is a promising source of functional foods, cosmetics and pharmaceuticals.

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