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Lipase Catalyzed Synthesis of Neutral Glycerides Rich in Micronutrients from Rice Bran Oil Fatty Acid Distillate

Sumit Nandi[1], Sarbani Gangopadhyay[1] and Santinath Ghosh[2]*

[1]*Narula Institute of Technology (81, Nilgunj Road, Agarpara, Kolkata-700 109, INDIA)*
[2]*Department of Chemical Technology, University of Calcutta (92, A.P.C.Road, Kolkata-700009, West Bengal, INDIA)*

Abstract: Neutral glycerides with micronutrients like sterols, tocopherols and squalene may be prepared from cheap raw material like rice bran oil fatty acid distillate (RBO FAD). RBO FAD is an important byproduct of vegetable oil refining industries in the physical refining process. Glycerides like triacylglycerols (TAG), diacylglycerols (DAG) and monoacylglycerols (MAG) containing significant amounts of unsaponifiable matter like sterols, tocopherols and hydrocarbons (mainly squalene) may certainly be considered as novel functional food ingredients. Fatty acids present in RBO FAD were esterified with glycerol of varying amount (1:0.33, 1:0.5, 1:1 and 1:1.5 of FAD : glycerol ratio) for 8 h using non-specific enzyme NS 40013 (*Candida antartica*). After esterification the product mixture containing mono, di- and triglycerides was purified by molecular distillation to remove excess free fatty acids and also other volatile undesirable components. The purified product containing sterols, tocopherols and squalene can be utilized in various food formulations.

Key words: fatty acid distillate; *Candida antartica,* rice bran oil, squalene, tocopherols, sterols, monoglyceride

1 INTRODUCTION

During physical refining of fats and oils, fatty acid distillate (FAD) is produced as a by-product, which is mainly utilized in soap manufacturing process. Rice bran oil (RBO) FAD is a major byproduct of RBO refining industries. RBO FAD contains higher amount of unsaponifiable matters amongst which sterols, tocopherols and hydrocarbons (mainly squalene) are main components. RBO FAD can be converted to neutral glycerides containing monoacylglycerol (MAG), diacylglycerol (DAG) along with these unsaponifiable matters. MAGs are the most widely used emulsifiers in food, pharmaceutical and cosmetic industries[1,2]. Owing to their ability to form complexes with amylose or pecteins, MAGs are also used as conditioning agents to modify starch- and protein-containing products. In addition, they also can be utilized to modify physical characteristics of fats by controlling their crystal polymorphism[3], or as building block for the synthesis of structured TAG[4-6]. MAG can be converted to structured glycerides by esterification with selective fatty acids like short chain and higher polyunsaturated fatty acids[7,8].

Diacylglycerol occurs naturally as a minor component of various edible fats and oils[9] and has been used in foods as an emulsifier. DAG has been utilized as a cocoa butter anti blooming agent[10] and as an intermediate in the synthesis of structured lipids[11,12]. Recent studies on the nutritional properties and dietary effects of DAG[13-18] have revealed that DAG, of which 1, 3-DAG is a major component, in contrast to TAG, had the ability to reduce serum TAG concentrations and as a result, to decrease both body weight and visceral fat mass in rats and humans[19,20].

Tocopherols and tocotrienols present in RBO can improve the blood circulation and reduce the oxygen demand of human body. α-tocopherol is used in pharmaceutical and cosmetic industries and a mixture of α-, γ- and δ-tocopherols is added to various foods including fats and oils. Phytosterols are known to interfere with the intestinal absorption of cholesterols and to reduce the cholesterol content in the blood[21-24]. It also acts as a natural anti-oxidant that protects the occurrence of rancidity in the oil without any synthetic antioxidant. Squalene has the ability to assist the skin in retaining moisture. Squalene

*Correspondence to: Santinath Ghosh, Department of Chemical Technology, University of Calcutta, 92, A.P.C.Road, Kolkata-700009, West Bengal, INDIA
E-mail: santinathghosh@yahoo.com.hk
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helps to keep our skin soft and healthy and its antioxidant capabilities help to protect from the harsh effects of the environment. Squalene is being investigated as an adjunctive therapy in some cancers[25], effective in inhibiting lung tumors and also demonstrated chemo protective effects against colon cancer in animal models[26].

In the present study, RBO FAD containing about 15% unsaponifiable matters, rich in sterols, tocopherols and squalene, is being utilized to produce different glycerides using lipase catalyzed reaction in a solvent free system.

2 MATERIALS AND METHODS

2.1 Materials

Rice bran oil FAD was obtained from Sethia Oil Mill, Burdwan, West Bengal, India. Glycerol (A.R.) was purchased from E. Merck (India) Pvt. Limited. The lipase NS 40013 (*Candida antartica*, a non-specific, immobilized lipase) was a kind gift of Novozymes, South Asia Pvt. Ltd., Bangalore, India.

2.2 Bleaching of fatty acid distillate

About 500 g rice bran FAD was taken in a 1 L round bottomed flask and heated under vacuum (2-4 mm Hg pressure) in a boiling water bath with shaking for 15 min. Then 4% Tonsil earth (Sud Chemical Company, Germany) and 0.5% activated charcoal (E. Merck, India, Ltd.) was added and shaken vigorously for 20 min under vacuum. After that it was cooled to 50°C and filtered under vacuum. The bleached FAD was then stored in a refrigerator at −20°C for further study.

2.3 Enzymatic esterification reaction

The fatty acid distillate and glycerol in different proportions were taken in a round bottomed flask and stirred by a magnetic stirrer at 65 ± 2°C under reduced pressure of 4 mm Hg for 8 h using 5% (by weight of substrates) NS 40013 lipase. The esterification reaction was monitored by estimating the free fatty acid content in the reaction mixture periodically withdrawn. After 8 h of reaction time, the product mixture was filtered to remove the enzyme and isolated for purification.

2.4 Purification of the product

The enzyme free mixture was purified in a molecular distillation unit (SIBATA Scientific Co. Ltd., Japan, Model MS–300) at 140-145°C temperature and 15-pascal pressure to remove residual free fatty acids along with some volatile impurities. It was a falling film type apparatus and was provided with a rotating wiper that continuously rubbed the falling film on the evaporating surface. The neutral glycerides (mono-, di- and triacylglycerol) were determined by standard column chromatographic IUPAC method[27].

2.5 Gas chromatographic analysis

Fatty acid composition was determined by a gas-liquid chromatographic (GLC) method after converting into methyl esters. The HP-5890A GLC was connected with a HP-3390A data integrator. The GLC was fitted with a glass column (1.83 m × 3.175 mm i.d) packed with 10% DEGS supported on Chromosorb-WHP (100/200 mesh), of HP make. The oven temperature was programmed from 100 to 190°C at 5° per min. The injector and detector block temperatures were maintained at 230 and 240°C, respectively. IOLAR-2 nitrogen was used as the carrier gas (flow rate 30 mL/min). The fatty acid ester peaks were identified and calibrated with standard methyl esters. Data were represented an averages of three determinations.

2.6 Determination of total tocopherol by colorimetric method (Emerie-Engel method)

Total tocopherol content in FAD and final products was measured according to the standard IUPAC method of Emerie-Engel[27].

2.7 Determination of sterols and squalene by HPLC method

Sterols and squalene were determined by HPLC methods. The HPLC instrument (Waters, USA) was provided with Binary HPLC Pump 1525 and Waters Dual Absorbance UV Detector 2487 and Refractive Index Detector 2414. The HPLC column (4.6 × 155 mm) was Novapak bonded C18 having micro particulate silica of particle size of about 5 μm. The isocratic flow rate was 0.5 mL/min. The whole system was supported by Breeze 2000 software.

For detection of sterols and squalene the mobile phase consisted of HPLC grade hexane, acetonitrile and isopropyl alcohol in the ratio of 75:15:10 v/v. The UV detector was used at 210 nm for squalene and 230 nm for sterols. The unsaponifiable matters of FAD were determined by using standard IUPAC method[27]. Then approximately 1.0 mg unsaponifiable matter was dissolved in HPLC grade hexane and was filtered through a Millipore filter. 10 μL of the solution was injected and the materials were detected according to their retention time and quantified with reference to the standard samples.

3 RESULTS AND DISCUSSION

Table 1 shows the total fatty acid composition, unsaponifiable matter and neutral glycerides present in RBO FAD. RBO FAD contains 77% free fatty acids and 7.9% neutral glycerides of which 1.2% MAG, 2.1% DAG and 2.6% TAG. The fatty acid composition is similar with that of standard ricebran oil, which is obvious. On the contrary, FAD contains high amount of unsaponifiable matter (15%) of which squalene, tocopherols and sterols are present at

Table 1 Analytical Characteristics and Fatty Acid Composition of Rice Bran Oil Fatty Acid Distillate.

Properties	RBO FAD (%, w/w)
Free fatty acid	77.1
Neutral glycerides	
Monoacylglycerol (MAG)	1.2
Diacylglycerol (DAG)	2.1
Triacylglycerol (TAG)	4.6
Fatty acid composition	
C16:0	28.4
C18:0	1.2
C18:1	37.2
C18:2	33.2
Unsaponifiable matter	15.0
Hydrocarbons	53.1
Tocopherols	32.1
Sterols	14.8

41.5, 32.1 and 14.8% respectively. RBO FAD was bleached thoroughly to remove the peroxides (at 1-2 Meq/kg level) before processing by enzymatic method.

For the production of neutral glycerides rich in micronutrients, the RBO FAD is esterified with glycerol in different proportions using non-specific NS-40013 lipase. FAD and glycerol concentration were maintained at 1:0.33, 1:0.5, 1:1 and 1:1.5 to study the esterification reaction for product-I (P-I), product-II (P-II), product-III (P-III) and product-IV (P-IV) respectively. Figure 1 demonstrates the time course of esterification reaction for 8 h. With the increase of glycerol concentration, the residual FFA in the reaction mixture was drastically reduced. After 8 h reaction time the product mixture contains 13.2, 12.5, 8.5 and 2.1% FFA in products I-IV respectively. The compositions of the esterified products are shown in Table 2. P-I, P-II, P-III and P-IV contained 42.5, 14.1, 7.2 and 4.4% TAG; 31.9, 53.8, 31.2 and 20.7% DAG and 10.2, 18.4, 48.7 and 62.8% MAG respectively. Also P-I, P-II, P-III and P-IV contained 15.4, 13.7, 12.9 and 12.1% unsaponifiable matters respectively.

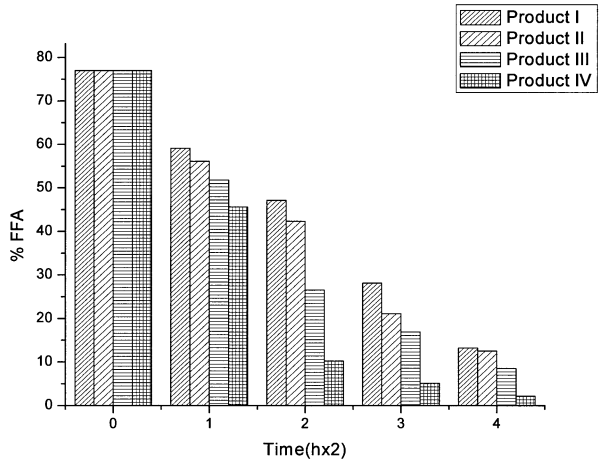

Fig. 1 Enzymatic Glycerolysis of Rice Bran Oil FAD for Neutral Glycerides.
Enzyme used: non-specific lipase NS 40013 (*Candida antartica*); Temperature: 60 ± 2 C
Ratio of fatty acid to glycerol
i) Product I : Fatty acid: Glycerol: : 1: 0.33
ii) Product II : Fatty acid: Glycerol: : 1: 0.5
iii) Product III : Fatty acid: Glycerol: : 1:1
iv) Product IV : Fatty acid: Glycerol: : 1:1.5

From Table 2, it can be concluded that among the four neutral glycerides, P-I can be considered as TAG rich (TAG -42.5%), P-II can be considered as DAG rich (DAG-53.8%) and P-IV can be considered as MAG rich (MAG-62.8%) glycerides. Simply varying the glycerol concentration in the reaction mixture the product composition can be changed and products of desired composition can be designed. The Kao Co., Tokyo, Japan has already marketing the healthy Econa brand oil containing 80% DG. So, the product no. II containing 53.8 % DG along with other micronutrients can be of commercial value.

Table 3 shows the composition of unsaponifiable matters of neutral glycerides. Product I, II, III and IV contained 30.5, 29.3, 29.1 and 28.7% tocopherols; 13.8, 12.8, 12.6 and 12.5% sterols and 52.9, 55, 55.5 and 55.8% squalene respectively. These specific glyceride rich products along with

Table 2 Composition of the Neutral Glycerides.

PRODUCT NO	TAG	DAG	MAG	UNSAP. MATTER
I	42.5 ± 0.75	31.9 ± 0.46	10.2 ± 0.28	15.4 ± 0.48
II	14.1 ± 0.33	53.8 ± 0.63	18.4 ± 0.54	13.7 ± 0.64
III	7.2 ± 0.08	31.2 ± 0.30	48.7 ± 0.72	12.9 ± 0.48
IV	4.4 ± 0.04	20.7 ± 0.21	62.8 ± 1.52	12.1 ± 0.89

TAG; Triacylglycerol, DAG-Diacylglycerol, MAG-onoacylglycerol
Values are represented as mean ± S.D. n=3

Table 3 Composition of the Unsaponifiable Matters of the Neutral Glycerides.

Products	Tocopherols (Total) (%w/w)	Sterols (%w/w) (Total)	Squalene (%w/w)	Others (%w/w)
I	30.5 ± 1.36	13.8 ± 0.09	52.9 ± 0.69	2.8 ± 0.12
II	29.3 ± 1.6	12.8 ± 0.25	55.0 ± 0.61	2.9 ± 0.14
III	29.1 ± 0.65	12.6 ± 0.24	55.5 ± 0.59	2.8 ± 0.13
IV	28.7 ± 0.43	12.5 ± 0.26	55.8 ± 0.63	3.0 ± 0.13

Values are represented as mean ± S.D. n=3

Table 4 Quality Parameters of the Final Products of Neutral Glycerides.

Products	Acid Value	Anisidine Value	Peroxide Value (meq/kg)	Colour (Lovibond 1″ cell)
I	< 0.1	0.4 ± 0.01	< 1	1.2Y + 1.2R
II	< 0.1	1.1 ± 0.04	< 1	1.9Y + 0.9R
III	< 0.1	1.1 ± 0.12	< 1	1.8Y + 0.7R
IV	< 0.1	1.2 ± 0.13	< 1	1.3Y + 0.9R

Values are represented as mean ± S.D. n=3

micronutrients are useful for functional food applications.

Table 4 shows the quality of micronutrient rich neutral glycerides on the basis of acid value, peroxide value, anisidine value and colour. By efficient molecular distillation at high vacuum it has been possible to reduce the FFA content and other volatile odoriferous compounds to a minimum level. The final products have acid value <0.1, peroxide value <1, anisidine value 0.4-1.2 with acceptable range of colour. So the purified products can be utilized for commercial exploitation. However, further studies are required on the stability of the final products.

4 CONCLUSION

Neutral glycerides containing significant amount of sterols, tocopherols and squalene can be produced from RBO FAD by lipase catalyzed esterification reaction. Thus, microbial lipase technology may be suitable in producing better quality of the concerned products from the relatively inferior grade raw materials.

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