Enzyme-Catalyzed Synthesis and Characterization of Octyl Dihydroxystearate from Palm-Based Dihydroxystearic Acid

Roila AWANG^{1*}, Mahiran BASRI², Salmiah AHMAD¹ and
Abu Bakar SALLEH²

¹ Advanced Oleochemical Technology Centre, Malaysian Palm Oil Board
(43650 Bandar Baru Bangi Selangor, MALAYSIA)

² Enzyme and Microbial Technology Research, Fakulti Sains & Pengajian Alam Sekitar, Universiti Putra Malaysia
(43400 Serdang, Selangor, MALAYSIA)

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Abstract: Octyl dihydroxystearate was synthesized enzymatically to overcome the problems associated with chemical processes such as high energy costs and degradation of ester. Immobilized enzyme, Lipozyme IM was employed as catalyst in the esterification reaction between dihydroxystearic acid (DHSA) and 1-octanol. The product obtained was characterized by chromatographic and spectroscopic methods (thin-layer chromatography, gas chromatography, fourier transform infrared, nuclear magnetic resonance spectroscopy) as well as wet chemistry. The crude product has a melting point of 59.0°C, saponification value of 140.5 mgKOH/g, hydroxyl value of 223.9 mgKOH/g and acid value of 7.9 mgKOH/g. The yield was about 95% with respect to the amount of DHSA used. The product obtained was found to contain octyl dihydroxystearate, octyl palmitate and octyl stearate. This compound was found to be nonirritating.

Key words: octyl dihydroxystearate, dihydroxystearic acid, irritancy, characterization

1 Introduction

Wax esters are esters of long chain fatty acids and long chain fatty alcohols. They have a wide range of uses as lubricants, plasticizers, coating materials in the medical and food industries, and as raw materials in cosmetics and other chemical industries (1). Hydroxy stearates are usually known in the form of wax esters (2). Hydroxy acid esters have a variety of uses; such as plasticizers, mold release agent, cosmetics (hair products, lipsticks and lotion) and chemical intermediates (3). Sulphonated wax esters of ricinoleic acid are promising surface-active compound (4). Currently wax esters are produced by reacting a fatty acid and an alco-

hol at high temperatures in the presence of tin, titanium, or sulfuric acid catalyst. This high-temperature process can lead to degradation of the esters and undesired side reactions; additionally the resulting energy cost is high (5).

Taking these aspects into consideration, biotechnological methods, such as bioconversion, become more advantages. Lipase-catalyzed esterification is possible at moderate temperature at neutral pH under normal pressure in simple reaction vessels. Recently, lipases with organic solvent-based media have been employed for the synthesis of hydroxy acid esters. Goshay and Bhattacharyya (4) and Mukesh *et al.*, (6) have produced ricinoleic acid wax ester from *Rhizomucor meihei*

*Correspondence to: Roila AWANG, Advanced Oleochemical Technology Centre, Lot 9 & 11, Jalan P/14, Section 10, 43650 Bandar Baru Bangi, Selangor, MALAYSIA
E-mail: roila@mpob.gov.my

lipase-catalyzed esterification of ricinoleic acid or alcoholysis of castor oil at high yield. While Lang *et al.*, (7) synthesized wax esters of (*S*)-17-hydroxystearic acid and (*R*)-3-hydroxydecanoic acid with various lipases. Several hydroxy acid wax esters produced in these investigation were found to be useful surfactant (3, 4, 6, 7). Previous study had shown that lipase-catalyzed esterification of DHSA and monohydric alcohol could be achieved with high yield (8). However, there is no complete data on the characterization of octyl dihydroxystearate. Therefore, in this paper, we report the synthesis and characterization of octyl dihydroxystearate via direct esterification of dihydroxystearic acid and 1-octanol using Lipozyme IM as catalyst.

2 Materials and Methods

2.1 Materials

DHSA was prepared in the laboratory (9). 1-Octanol (purity, 99%) was purchased from Sigma Chemical Co. (St. Louis, MO). Lipozyme IM (lipase from *R. meihe*i was donated by Novozyme A/S (Bagsvaerd, Denmark). *N,O*-Bis-trimethylsilyl acetamide (purity, > 98%) was from Merck (Darmstadt, Germany). All other reagents were of analytical grade and used as received.

2.2 Octyl Dihydroxystearate Synthesis

In general, 1-octanol was used at a molar excess of 2:1 in 250-mL stoppered Erlenmeyer flask in order to increase solubility of DHSA in reaction mixture. The bioconversion was started by addition of 15% (w/w) Lipozyme IM and the reaction mixture consisted of DHSA (0.1 mol) and 1-octanol (0.2 mol) was incubated at 50°C for 3h with shaking speed of 200 rpm. Hexane was used as a solvent. For the purpose of further analysis, the reaction was terminated by separating the enzyme from the mixture by means of filtration using a filter paper. The solvent was removed by rotary evaporation. Details of product isolation and purification were described in our previous study (8).

2.3 Thin-Layer Chromatography (TLC)

Sample of crude product was dissolved in appropriate organic solvent and separated on a layer of silica gel with hexane/ethyl acetate/acetic acid (20:80:1, vol/vol/vol) as the developing solvent. Visualization of the chromatogram was carried out by charring at 140°C after the plate was sprayed with sulfuric acid solution

(50%).

2.4 Gas Chromatography (GC) of Trimethylsilyl (TMS) Derivatives of Octyl Dihydroxystearate

GC analysis was carried out using a Hewlett-Packard HP-6860A Plus gas chromatograph (Palo Alto, CA). The TMS derivatives of octyl dihydroxystearate was separated on a nonpolar column, HP-5 (Hewlett-Packard, 30 m × 0.25 mm × 0.25 (μm) with helium as the carrier gas. The oven was programmed to hold at 150°C for 1 min, followed by ramping from 150°C to 290°C at a rate of 10°C/min. The final temperature was held at 290°C for 30 min. The injector and flame-ionization detector were set at 300°C.

2.5 GC-Mass Spectrometry (GC-MS) of TMS-Derivatives of Octyl Dihydroxystearate

GC-MS of the TMS-derivatives was performed on a Perkin Elmer Instrument model Q-mass 910 mass spectrometry. The GC was equipped with a SE-52 capillary column. The carrier gas was helium at a flow rate of 1 mL/min. The column temperature was initially kept at 180°C for 1 min, then programmed to 280°C at 10°C/min. The final temperature was held for 35 min. Other operating conditions were; injector temperature of 280°C, auxiliary temperature 220°C and split ratio was 100:1.

2.6 Preparation of TMS-Derivatives for GC and GC-MS Analyses

Sample (0.01g) was weighed into a vial. One mL of GC grade *N,N*-dimethylformamide and *N,O*-bis-trimethylsilyl acetamide (0.5 mL) were added. The sample was mixed using vortex mixer for 15-30 sec and warmed at 60°C for 30 min. The sample was allowed to cool for a few minutes before injection.

2.7 Nuclear Magnetic Resonance (NMR) Spectroscopy of Octyl Dihydroxystearate

Proton NMR (¹H NMR) spectrum was obtained on a Hitachi NMR R-1200 spectrometer (60 MHz) in deuterated chloroform (CDCl₃) with tetramethylsilane as internal standard. ¹³C NMR spectrum was recorded on a Bruker DRX-300 (Karlsruhe, Germany) spectrometer at 300 MHz. The chemical shifts are expressed in ppm.

Acid value, iodine value, hydroxyl value and saponification value were determined by standard procedures (AOCS official methods): acid value, Te 1a-64; iodine value, Cd 1d-92; hydroxyl value, Cd I3-60; saponification value TI 1a-64 (10). Fourier transform infrared (FTIR) spectra were recorded on a Nicolet Magna-IR550 (Nicolet, Madison, WI) spectrophotometer.

2.8 Thermal Stability

Thermal stability of octyl dihydroxystearate was studied using a Differential Scanning Calorimeter (DSC) (TA, Instrument, New Castle, DE). Samples of 10 mg were weighed in ceramic measuring cup, which were closed and placed into the calorimeter. An empty cup with cap was used for reference. DSC heating curves were determined by heating at a temperature range from room temperature to 190°C at a heating rate of 5°C/min. The heating process was repeated 3 times.

2.9 Solubility Test

Sample (0.1g) was placed into a conical flask. Solvent was then added dropwise to the flask using a buret. Continuous stirring at room temperature was carried out until no undissolved particles were seen. The solution was stirred for 15 min before solubility was determined.

2.10 Irritancy Test

Dermal irritancy of octyl dihydroxystearate was conducted using The Irritation Assay System, which consists of a test kit, instrumentation, and computer software (In Vitro International, Irvine, CA). Samples were weighed at four different concentrations- 50, 75, 100 and 125 mg-and placed into the membrane discs. Reagent and blanking buffer (1250 μ L) were added to a 24-well assay plate. The membrane discs that con-

tained various concentrations of octyl dihydroxystearate sample were inserted into the corresponding blank and test sample wells of the plate. The assay plate was then incubated at 25°C for 24h. After this time the membrane discs were removed from the assay plate, and 250 μ L of reagent plus blanking buffer was transferred into the 96-well reading plate. This plate was inserted into the MRX Microplate Reader (Dynex Technologies, Inc., Chantilly, VA).

3 Results and Discussion

In a typical reaction of DHSA with 1-octanol, catalyzed by Lipozyme IM, the reaction product was isolated as described in the Experimental section, to yield of octyl dihydroxystearate of 95% with respect to the amount of DHSA used.

3.1 Analysis of Product

Properties of the starting materials and the products, such as hydroxyl value, saponification value, acid value and melting point are shown in **Table 1**. The results showed that the acid value decreased in the conversion of the reactant to the product. This indicates the formation of ester compound. The saponification value and hydroxyl value also decreased, this may be due to the increasing of molecular weight of the product.

TLC of the product indicates the formation of new product (**Fig. 1**). In the FTIR spectrum, a strong O-H transmittance peak appears at 3330 cm^{-1} , which indicates that the hydroxyl group of parent acid remains unaffected by action of the enzyme. The carboxylic carbonyl transmittance peak shifts from 1709 cm^{-1} to 1739 cm^{-1} in the product due to the change from acid to ester group. This finding was in agreement with Sil-

Table 1 Properties of DHSA, Crude Product and Purified Product^{a,b}.

Parameter	DHSA [*]	Crude Product	Purified Product
Acid Value (mgKOH/g)	180.3 $\pm$ 1.2	7.9 $\pm$ 0.8	1.8 $\pm$ 0.1
OHV (mgKOH/g)	309.3 $\pm$ 3.9	223.9 $\pm$ 4.6	246.1 $\pm$ 3.5
Sap. Value (mgKOH/g)	178.5 $\pm$ 1.0	140.5 $\pm$ 0.5	138.5 $\pm$ 0.7
Iodine Value (g I ₂ /100g)	1.1 $\pm$ 0.2	6.0 $\pm$ 0.1	0.3 $\pm$ 0.1
Melting Point (°C)	90.6 $\pm$ 0.9	59.0 $\pm$ 0.1	69.8 $\pm$ 0.2

^aPurified product obtained by column chromatography method.

^bAverage values determined in duplicate.

^{*}Data from Awang *et al.*, (16).

Fig. 1 Thin-layer Chromatogram of Octyl Dihydroxystearate Derived from Palm-based DHSA. Developing solvent: Hexane/ethyl acetate/acetic acid (20:80:1, by vol). (1) 1-octanol, (2) DHSA, (3) crude product, (A) product, (B) unreacted 1-octanol and (C) unreacted DHSA

vertein *et al.*, (11) who proposed that ester compound have a characteristically strong absorption band arising from C=O stretching at $1730\text{--}1740\text{ cm}^{-1}$.

3.2 GC Analysis

GC analysis of octyl dihydroxystearate was compared with the standard (data not shown). As shown in **Fig. 2**, the chromatogram of octyl dihydroxystearate after conversion to TMS derivatives revealed the presence of other compounds along with octyl dihydroxystearate. The major peak was appeared at retention time (Rt) 19.12 min assigned to octyl dihydroxystearate. Unreacted DHSA and 1-octanol appeared at Rt 14.33 min and 2.89 min, respectively. While octyl palmitate and octyl stearate at Rt 14.55 min and 16.09 min. These are possibly due to the trace impurities present in DHSA. The oleic acid used as starting materials for production of DHSA is of 75% purity. It also contained saturated fatty acid at various chain length (C_{12} , C_{14} , C_{16} , C_{18} and C_{20}) (12).

3.3 GC-Mass Spectrometry Analysis

Scheme 1 shows a general MS fragmentation pattern of octyl dihydroxystearate. The MS data for octyl dihydroxystearate showed that the major ions at m/z 358

Fig. 2 Gas Chromatographic Separation of the Components of Octyl Dihydroxystearate Prepared from Palm Based DHSA after Derivatization with *N,O*-bistrimethylsilyl Acetamide. Column: HP-5 [(5%-phenyl)-methylpolysiloxane], $0.25\text{ mm} \times 30\text{ m} \times 0.25\text{ }\mu\text{m}$. Oven: 150°C for 1 min, $150\text{--}290^{\circ}\text{C}$ at $10^{\circ}\text{C}/\text{min}$, 290°C for 30 min.

Scheme 1 General Fragmentation of Octyl Dihydroxystearate.

and 215 arise from path a and b cleavage (**Fig. 3**) whereas m/z 431 is expected rearrangement ion resulting from path c cleavage and TMS migration with loss of CHO (m/z 459-29). The fragmentation at m/z 319 confirms path d cleavage. Similar fragmentation pattern was reported by Carlson *et al.*, (13) for methyl 13,14-dihydroxydocosanoate.

3.4 NMR Analysis

Octyl dihydroxystearate was also subjected to ^1H NMR and ^{13}C NMR: ^1H NMR of octyl dihydroxystearate in CDCl_3 exhibited the following peaks: 4.07 ppm (OH), 3.96 ppm (CHOH), 2.73 ppm ($\text{CH}_2-\text{CH}(\text{O})$), 1.28-1.80 ppm (CH_2) and 0.94 ppm (CH_3). ^{13}C NMR confirmed the presence of the following group: carbonyl at 174.08 ppm, two hydroxyls carbon at 74.47 ppm and 74.66 ppm, $-\text{O}-\text{CH}_2$ at 64.48 ppm, $-\text{CH}_2-$ carbon cover the range from 24.96 to 34.37 ppm, two $-\text{CH}_2\text{CH}_3$ groups at 22.68 and 22.65 ppm and methyl carbon at 14.12 ppm (8).

3.5 Thermal Stability

Thermal stability of crude and purified octyl dihydroxystearate was determined based on melting point by using DSC. The DSC melting thermograms of crude octyl dihydroxystearate revealed a variation in melting point from 63.7°C to 66.8°C after being heated at RT to 190°C for 3 cycles (**Fig. 4**). However, there was no variation in the melting point for purified product (**Fig. 5**).

3.6 Solubility Properties

As shown in **Fig. 6** crude octyl dihydroxystearate is more soluble in alcohol compared to purified product. This observation may be due to the presence of excess octanol in crude product, where octanol was removed during purification process. The solubility of this compound is increased by increasing alcohol chain-length from C_1 to C_3 and slightly decreased or constant at higher chain-length. This phenomenon may be attributed to increase in viscosity of solvent.

According to Kim (14) the solubility of a substrate in a nonaqueous system depends primarily on the melting point and heat of fusion of the solute and on those characteristic of the solvent that determine the degree of mutual affinity of solvent and solute molecules.

3.7 Irritancy Properties

Table 2 shows the dermal irritancy potential of octyl dihydroxystearate from various ratio of 1-octanol. All samples were predicted to be nonirritating with Human Irritancy Equivalent (HIE) score below 0.90. This results showed the skin compatibility of octyl dihydroxystearate. Therefore, it has potential for use in cosmetics

Fig. 3 Electron Impact GC-mass Spectrometry Fragmentation of Octyl Dihydroxystearate Derived from Palm Based DHSA after Derivatization with *N,O*-bistrimethylsilyl Acetamide.

Fig. 4 Differential Scanning Calorimetry Melting Thermogram of Crude Octyl Dihydroxystearate. Sample was heated at room temperature to 190°C at a rate of 5°C/min for 3 cycles.

Fig. 5 Differential Scanning Calorimetry Melting Thermogram of Purified Octyl Dihydroxystearate. Sample was heated at room temperature to 190°C at a rate of 5°C/min for 3 cycles.

Fig. 6 Solubility of Crude and Purified Octyl Dihydroxystearate in Alcohol of Various Chain Length. Solubility test was carried out as described in Materials and Methods Section. Viscosity was measured at 25°C using CP40 spindle at 2.5 rpm.

Table 2 Irritancy Test of Octyl Dihydroxystearate Produced at Various Mole Ratio of Substrate^{a,b}.

Dose, mg	Irritancy score (DHSA: 1-Octanol)			Irritancy Classification
	1.0 : 1.0	1.0 : 1.5	1.0 : 2.0	
50	0.28	0.22	0.00	Nonirritant
75	0.25	0.28	0.05	Nonirritant
100	0.37	0.29	0.16	Nonirritant
125	0.60	0.33	0.25	Nonirritant

^aData were from single analyses.

^bData were obtained using The Irritation Assay System (In Vitro International, Irvine, CA) as described in the Materials and Methods section of the test.

and personal care products. It was reported that the Dermal Irritation Assay Method could be employed to evaluate multiple samples at varying concentrations readily. Thus the test serves as a useful screening tool that facilitates all stages of raw material selection, formulation development, and final product selection (15).

4 Conclusion

Octyl dihydroxystearate was synthesized by Lipzyme IM at mild condition with high percentage of conversion. This compound was found to be nonirritating.

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References

1. M.L. HALBERG, D. WANG and M. HARROD, Enzymatic Synthesis of Wax Ester from Rapeseed Fatty Methyl Esters and Fatty Alcohol, *J. Am. Oil Chem. Soc.*, Vol. **76**, 183-187 (1999).
2. M. GOSH and D.K. BHATTACHARYYA, Lipase-Catalyzed Synthesis of Hydroxystearated and Their Properties, *J. Am. Oil Chem. Soc.*, Vol. **75**, 1057-1059 (1998).
3. D.G. HAYES and R. KLEIMAN, Lipase-Catalyzed Synthesis of Lesquerolic Acid Wax and Diol Esters and Their Properties, *J. Am. Oil Chem. Soc.*, Vol. **73**, 1385-1392 (1996).
4. S. GHOSHRAJ and D.K. BHATTACHARYYA, Enzymatic Preparation of Ricinoleic Acid Esters of Long-Chain Monohydric Alcohols and Properties of the Esters, *J. Am. Oil Chem. Soc.*, Vol. **69**, 85-88 (1992).
5. W. LOKOTSCH, S. LANG, D. MOBIUS and F. WAGNER, Biocatalytical Synthesis and Monolayer Studies of Multiple Hydroxylated Wax Esters, *J. Am. Oil Chem. Soc.*, Vol. **73**, 1459-1464 (1996).
6. D. MUKESH, R.S. IYER, J.S. WAGH, A.A. MIKASHI, A.A. BANERJI, R.V. NEWADKAR and H.S. BEVINAKATTI, Lipase Catalyzed Transesterification of Castor Oil, *Biotechnol. Lett.*, Vol. **15**, 251-256 (1996).
7. S. LANG, R. MULTZSCH, A. PASSERI, A. SCHMEICHEL, B. STEFFEN, F. WAGNER, D. HAMANN and H.K. CAMMENGGA, Unusual Wax Esters and Glycolipids: Biocatalytical Formation and Physico-Chemical Characterization, *Acta Biotechnol.*, Vol. **11**, 379-386 (1996).
8. R. AWANG, M. BASRI, S. AHMAD and A.B. SALLEH, Enzymatic Esterification of Dihydroxystearic Acid, *J. Am. Oil Chem. Soc.*, Vol. **77**, 609-612 (2000).
9. R. AWANG, S. AHMAD and Y.B. KANG, *Malaysian Patent PI* 9, 804,456 (1998).
10. *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th Edn., AOCS Press, Champaign (1995).
11. R.M. SILVERSTEIN, G.C. BASSLER and T.C. MORILL, *Spectrometric Identification of Organic Compounds*, 5th edn., John Wiley & Son, New York, pp. 122-124 (1991).
12. R. AWANG and S. AHMAD, *Proceeding of 2001 International Palm Oil Congress-Oleochemicals conferences*, Malaysian Palm Oil Board, Bangi, Kuala Lumpur, Malaysia, pp. 90-96 (2001).
13. K.D. CARLSON, R. KLEIMAN and M.O. BAGBY, Epoxidation of Lesquerella and Limnanthes (Meadowfoam) Oils, *J. Am. Oil Chem. Soc.*, Vol. **71**, 175-182 (1994).
14. S.J. KIM, *Cosmetic Raw Material Analysis and Quality, Vol. 1-Hydrocarbons, Glycerides, Waxes and other Esters* (Hilda Buttlar, ed.), Micelle Press, Weymouth, England, pp. 33-52 (1994).
15. Irritaction® Assay System Instruction Manual, In Vitro International, Irvine, CA, pp. 1-11 (1996).
16. R. AWANG, S. AHMAD, Y.B. KANG and R. ISMAIL, Characterization of Dihydroxystearic Acid from Palm Oleic Acid, *J. Am. Oil Chem. Soc.*, Vol. **78**, 1249-1252 (2001).