Scientific Research Publishing

Advances in Enzyme Research, 2016, 4, 152-157
http://www.scirp.org/journal/aer
ISSN Online: 2328-4854
ISSN Print: 2328-4846

Lipase Catalyzed Methanolysis of Tri-(12-Hydroxy Stearoyl)-Glycerol in Organic Solvents

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How to cite this paper: Kang, S.-T. and McKeon, T.A. (2016) Lipase Catalyzed Methanolysis of Tri-(12-Hydroxy Stearoyl)-Glycerol in Organic Solvents. *Advances in Enzyme Research*, **4**, 152-157.
http://dx.doi.org/10.4236/aer.2016.44014

Received: October 1, 2016
Accepted: December 25, 2016
Published: December 28, 2016

Abstract

Castor oil is the source of numerous products and is the only commercial source of the fatty acid ricinoleate, 12-hydroxy-oleate. Hydrogenated castor oil is similarly useful as the source of 12-hydroxy-stearic acid, best known as a component of lithium grease. Mono- and diacylglycerols are derived from castor oil and are useful in development of lubricants and emulsifiers for cosmetics, pharmaceutical and food use. Acylglycerols derived from hydrogenated castor oil may be similarly useful, albeit with different physical and chemical properties. We have evaluated the use of immobilized lipases to generate acylglycerols, using organic solvents to modulate the action of lipase to produce mono- and diacylglycerols, using tri-(12-hydroxy stearoyl)-glycerol as a model for hydrogenated castor oil. The presence of an alkylated oxygen in the solvent appears to be an important factor in supporting lipase activity, with diisopropyl ether providing the best yield of di-(12-hydroxy stearoyl)-glycerol.

Keywords

Castor Oil, Enzyme, Lipase, Diacylglycerol, Monoacylglycerol

1. Introduction

Castor oil is unique among commodity seed oils as it contains up to 90% ricinoleate (12-hydroxy-octadec-*cis*9-enoate). The presence of the mid-chain hydroxyl group imparts physical and chemical functionality making castor oil uniquely useful as a chemical and industrial feedstock. Castor oil forms the basis for emulsifiers used in food, cosmetic and pharmaceutical preparations. Acylglycerols and free fatty acids derived from hydrogenated castor oil contain 12-hydroxy stearate, and these products are similarly useful as emulsifiers. The lithium salt of 12-hydroxy stearate is used in formulating

the well-known lubricant lithium grease which is very useful for reducing metal-on-metal friction. The hydroxyl and carboxyl groups interact with metal and the non-polar hydrocarbon backbones help to retain non-polar additives. Similar to ricinoleate, the hydroxyl-stearate is also used as an emulsifier in cosmetics and adds beneficial antimicrobial activity to help maintain product stability [1].

We have previously demonstrated the utility of lipases for extraction from seed and simultaneous transmethylation using supercritical CO_2 extraction with methanol as a co-solvent on a bed of immobilized lipase [2]. Additionally, by judicious use of solvent and lipase, we have been able to produce high yields of 1,2-diricinoleoyl diacylglycerol [3]. The 1,2-diricinoleoyl DG was especially useful in identifying the activity of the diacylglycerol acyltransferase from castor seed [4]. By varying the amount of hydroxyl fatty acid substitution on glycerol, in addition to their use as emulsifiers and viscosity modulators, we believe these products may also be of value in developing polyurethanes and composites with differing functional properties resulting from the differences in acyl-substitution on glycerol [1].

2. Materials and Methods

2.1. Materials

Solvents were purchased: HPLC-grade methanol, n-butanol, acetonitrile, acetone, ethyl acetate, chloroform, hexane and toluene from Fisher Scientific (Fairlawn, NJ); DIPE from FlukaChemie (Buchs, Switzerland); iso-octane from Sigma-Aldrich (St. Louis, MO) and ethanol from Acros Organics (Fairlawn, NJ). Lithium chloride, magnesium nitrate, sodium chloride and anhydrous sodium sulfate were obtained from J.T. Baker Inc. (Phillipsburg, NJ). Potassium sulfate, sodium phosphate, and disodium phosphate were purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA). The lipase R (P. roquefortii, PRL) was the kind gift from Amano Enzymes Inc. (Nagoya, Japan) and Lipozyme RMIM 60 from Rhizomucor miehei was generously supplied by Novozymes North America (Franklinton, NC).

THS-glycerol is not commercially available and was prepared by lipase-catalyzed esterification. Reactions were carried out by mixing 40.8 mg of glycerol adsorbed on silica gel (1:1, w/w) and 0.2 g of the 12-hydroxystearic acid (12-HSA) for a molar ratio of FFA to GL of 3:1. The reaction was performed at 85˚C, just above the melting temperature of 12-HAS. At this temperature no organic solvent is necessary to solubilize the substrates, which allows for the use of a reaction medium solely composed of the necessary substrates. The optimal conditions for synthesis of THS-glycerol were a molar ratio of the HS to adsorbed GL was 3:1, lipase RMIM loading was 10% (w/w) of the weight of HS, A_w for all components was adjusted to 0.11 by equilibration in a desiccator containing saturated LiCl [5], and the reaction temperature was 85˚C. The reaction mixture in an open glass vial (17 mm i.d. × 85 mm L) was mixed vigorously by a magnetic stirrer (150 rpm) at the equilibrated temperature of 85˚C. During incubation at different times, two 40 µl samples were withdrawn from each flask and mixed with 0.4 ml of 2-propanol and stored in a freezer at –20˚C for later HPLC analysis.

2.2. HPLC Analysis

The course of esterification was monitored by reverse phase HPLC as previously described [3]. The expected products of lipase reaction, *i.e.*, HS, MHS-glycerol, DHS-glycerol and THS-glycerol were analyzed by injection of 50μL of sample on a C_{18}RP-HPLC column, using mobile phase of methanol (A) and methanol: water (90:10) (B). The gradient was from 100% B to 100% A in 20 min, then held for 12min, with a reverse gradient to 100% B in 2 min, and equilibration with B for 6 min. The total run time was 40 min. The flow rate was 1.0 mL/min., and detection was performed at 205 nm. Results are expressed as percentage of peak areas. Retention times for MHS-glycerol, HS, DHS-glycerol and THS-glycerol were 4.94 min, 7.52 min, 20.46 min, and 32.34 min, respectively, and identity of each product confirmed by LC/MS.

Preparation of THS-glycerol for methanolysis reactions was carried out by combining the product from multiple reactions and isolating the THS-glycerol by preparative HPLC using a 250 × 15 mm Phenomenex Luna C_{18}RP column, 5 micron particle size, from Phenomenex, (Torrance, CA) eluted according to Turner *et al.* [3].

2.3. Methanolysis Reaction

General reaction conditions used were 100 mg of THS-glycerol (110micromole) dissolved in 5 ml of reaction media and 0.625 ml (1.54 mmole) of methanol was mixed with 100 mg of free enzyme. The lipase PRL, reaction media, methanol and glassware were allowed to equilibrate for at least 48h in desiccators containing a saturated aqueous solution of $MgNO_3$ to reach A_w of 0.53 [5]. The reaction took place in closed glass tubes at 25˚C and shaking at 220 rpm. Fractions of 100 microliter were taken out at pre-determined time intervals, the solvent was removed by nitrogen, 0.5 mL of 2-propanol was added to the residue and samples were stored briefly at −20˚C and analyzed by HPLC. The reactions were performed in duplicate.

3. Results and Discussion

Figure 1 displays the results of the esterification reaction of HS with glycerol as catalyzed by lipase RMIM. The reaction presented provided conditions needed to obtain a good yield of THS-glycerol and was not monitored closely following determination that, after a 1-day incubation, up to 80% of the product is THS. As an alternative to this lipase-catalyzed reaction, it is also possible to hydrogenate castor oil and isolate the THS-glycerol product by preparative HPLC as described in Materials and Methods.

The results of the methanolysis reaction (**Table 1**) show no clear connection with hydrophobicity of the solvent. Organic solvents provide a very useful medium for lipase-mediated action, as both substrate and product are hydrophobic and soluble in organic solvents [6] [7]. In general, enzyme reactions are considerably slower in organic solvents [8], although this is highly dependent on the substrate [9] with water-insoluble triacylglycerols more readily lysed by the action of lipases.. The *Penicillium* lipases tend to be less active on diacylglycerol substrates, thus being very useful for generating desired diacylglycerols as intermediates instead of monoacylglycerols or complete hy-

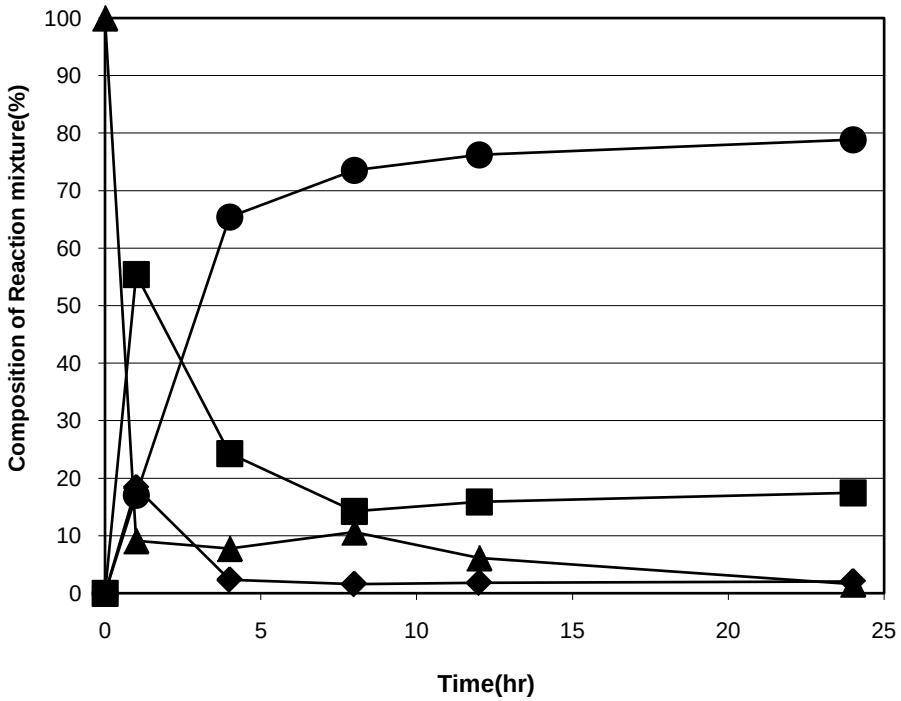

Figure 1. Production of triHS-glycerol by enzymatic esterification of 12-hydroxystearic acid and glycerol at Aw = 0.11; Reaction conditions were as follows: 40.8 mg of GL adsorbed on silica gel (weight ratio of glycerol to adsorbent = 1:1), 0.2 g of 12-hydroxystearic acid, molar ratio of FFA to glycerol of 3:1, 20 mg of RMIM (Aw 0.11), stirred at 150 rpm and 85˚C. MHS-glycerol (♦♦); HS (▲); DHS-glycerol (▪); and THS-glycerol (●).

Table 1. Effect of solvent on acyl-glycerol products from of tri-(12-hydroxy-stearate)-glycerol.

Solvents	Log P*	MHS-glycerol	DHS-glycerol	THS-glycerol	% Conversion to MHS- and DHS-glycerols
Acetonitrile	−0.34	2.0	24.4	59.6	31
Ethanol	−0.31	12.2	33.1	39.2	54
Acetone	−0.24	2.2	18.0	64.4	24
Ethyl acetate	0.73	2.2	17.4	65.3	23
1-Butanol	0.88	10.0	32.7	42.5	50
Diisopropyl ether	1.52	6.2	52.0	22.2	72
Chloroform	1.97	2.0	12.5	69.3	17
Toluene	2.73	7.5	28.6	48.0	43
Hexane	4.0	2.1	2.2	85.4	5
Isooctane	4.5	4.1	2.6	70.0	9

*From Sangster [6] except isooctane, from Kumar and Gross [7].

drolysis. In our results presented in Table 1, we see primarily an accumulation of diacylglycerols (DHS) in organic solvents with the exception of the two alcohols.

Gagnon and Vasudevan [10] observed a good correlation of lipase-catalyzed methanolysis of soybean oil with increasing hydrophobicity of the solvent used. Our results differ, but this is probably due to our use of a different lipase, PRL, different reaction

conditions and the unusual hydroxyl fatty acid. The higher levels of methanolysis are observed in the ether DIPE and the two alcohols, ethanol and butanol. Of course, the latter two solvents may also participate in the alcoholysis reaction and, aside from the acylglycerols, we did not attempt to quanify the levels of the fatty acid esters formed from the alcohol solvents. Similar results were observed, also using immobilized PRL to generate diacylglycerols from tricaprin, trilaurin and tripalmitin [9]. Methanolysis in toluene was similar in yield to the two alcohols and DIPE proved to be the best solvent for controlled methanolysis of THS with a yield of 73% MHS and DHS, and 65% DHS.

Generally, carefully controlled reactions require maintenance of stable water activity, as enzymes vary on their reactivity and stability in organic solvents as a result of the presence of water. Water will affect the structure of the enzyme and excessive water produced during hydrolysis can interfere with methanolysis as a result of the competing hydrolytic reaction [11].

4. Conclusion

Our results suggest that careful use of lipase in DIPE can provide suitable yields of DHS for use in emulsifiers for cosmetics, pharmaceuticals and food as well as viscosity modifiers in lubricants. The presence of two fatty acid hydroxyl groups and an available hydroxyl on the glycerol backbone provide the possibility of using these 12-hydroxy stearioyl diacylglycerols for developing polyurethanes with novel properties.

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Abbreviations

HS: 12-Hydroxystearate

DIPE: Diisopropyl Ether

MHS-glycerol: Mono-(12-hydroxy stearoyl)-glycerol

DHS-glycerol: Di-(12-hydroxy stearoyl)-glycerol

THS-glycerol: Tri-(12-hydroxy stearoyl)-glycerol

RP: Reverse-phase