Enzymatic Esterification of Dihydroxystearic Acid

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ABSTRACT: Dihydroxystearic acid (DHSA) ester was synthesized enzymatically to overcome the problems associated with chemical processes. Immobilized enzyme, Lipozyme IM and Novozym 435, were employed as catalysts in the esterification reaction between DHSA and monohydric alcohol. Various factors that may affect the esterification reaction were studied, such as initial water content (a_w), organic solvent, substrate concentration and the influence of alcohol chain length. It was found that the percent conversion was higher in organic solvents with log *P* (the logarithm of the partition coefficient of solvent in octanol/water system) from 2.0 to 4.0. The reaction was not affected by a_w from 0.09 to 0.96. Increasing the mole ratio of alcohol to acid above 2.0 did not increase the percent conversion of ester. The ester was identified by Fourier transform infrared and ^{13}C nuclear magnetic resonance spectroscopy.

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KEY WORDS: Alcohol, dihydroxystearic acid, esterification, lipase.

Hydroxy fatty acid esters are of considerable industrial interest compared with ordinary esters because of their different behaviors including solubility, surface-active properties, and thermal stability (1). These compounds have interesting applications, such as plasticizers, mold release agent, waxes, surfactant and chemical intermediates (2). Sulfonated wax esters of ricinoleic acid are promising surface-active compounds (3). Current manufacture of these esters is realized by reacting a fatty acid/hydroxy fatty acid and an alcohol at high temperature in the presence of a tin, titanium, or sulfuric acid catalyst for up 20 h. This high-temperature process can lead to degradation of the ester and undesired side reactions; additionally, the resulting energy costs are high (4).

Lipase-catalyzed reactions operate at mild conditions, which prevent degradation of starting materials and reduce side reactions. Application of lipases in various biochemical modifications of fats and oils is well established, and the catalytic activity of lipases toward hydroxy acids is also well studied by various workers (4–7). For example, *Rhizomucor miehei* lipase has been employed in the esterification of 12-hydroxystearic acid and alcohol (4). Mukesh *et al.* (5) have produced ricinoleic acid wax esters by *R. miehei* lipase-catalyzed esterification of ricinoleic acid or alcoholysis of castor oil. Wagner *et al*. (6) and Hayes (7) also employed *R. miehei* to prepare wax ester by esterification or alcoholysis reactions.

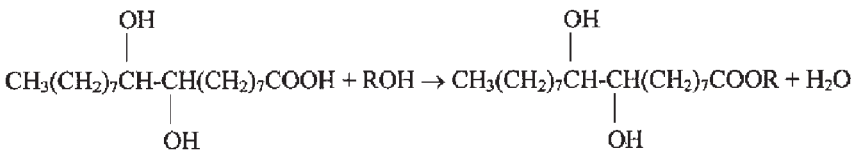
The aims of the present study are to investigate the enzymatic esterification reaction of the carboxyl group of dihydroxystearic acid (DHSA) with monohydric alcohol (Scheme 1) and to examine the effect of various reaction parameters.

MATERIALS AND METHODS

Materials. DHSA was prepared in the laboratory (8). 1-Octanol (purity, 99%) and 1-octadecanol (purity, 99%) were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Decanol (purity, 98%), 1-dodecanol (purity, 98%), 1-tetradecanol (purity, 98%), and 1-hexadecanol (purity, >95%) were from Merck (Darmstadt, Germany). Lipozyme IM (lipase from *R. miehei*) and Novozym 435 (lipase from *Candida antartica*) were donated by Novo Nordisk A/S (Bagsvaerd, Denmark). All other reagents were of analytical grade and used as received.

All experiments were carried out in triplicate and repeated twice. Results presented here are the average of these multiple determinations.

Esterification reaction. DHSA (2 mmol) and 1-octanol (4



SCHEME 1

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mmol) were placed in a 20-mL screw cap vial. *n*-Hexane (3.0 mL) was then added into the vial, followed by enzyme (0.3 g), and the vial was tightly closed. The mixture was incubated at 30°C in a horizontal water bath shaker with shaking speed of 200 rpm for 5 h. The reaction was carried out in triplicate and was repeated twice.

The reaction was terminated by dilution with acetone/ethanol (1:1 vol/vol, 3.5 mL). The unreacted free fatty acid in the reaction mixture was determined by titration with sodium hydroxide (0.05 M) to an end point of pH 10.00. For the blank determination, reaction mixture without enzyme was used. The percent conversion was calculated based on the unreacted free fatty acid in the reaction mixture using the following equation:

$$\% \text{ conversion} = [(B - A)/B] \times 100 \quad [1]$$

where A = amount of free fatty acid remaining in the sample mixture and B = amount of free fatty acid in the control sample.

Effect of water activity (a_w). The substrates and the enzyme were pre-equilibrated to known a_w in vapor phase overnight at room temperature (15). Salt hydrates used were P_2O_5 (a_w = 0.09), LiCl (a_w = 0.12), $MgCl_2 \cdot 6H_2O$ (a_w = 0.32), KI (a_w = 0.68), KCl (a_w = 0.86), and KNO_3 (a_w = 0.96). Then, the catalyst and the substrates were brought together and allowed to react by incubating the mixtures in a water bath shaker with shaking speed of 200 rpm for 5 h.

Solubility test. DHSA (0.1 g) was placed into a conical flask. Solvent was then added drop wise to the flask using a burette. Continuous stirring at 30°C was carried out until no particles were seen. The solution was stirred for 1 h before solubility was determined.

Isolation of the product. At the end of reaction period of 5 h, the reaction mixture was dissolved in diethyl ether. The enzyme was removed by filtration using a filter paper. The solvent was removed by rotary evaporation. The crude product was then subjected to column chromatography. The product was isolated by passing a known quantity of the crude product through a glass column (10 × 1.5 cm) packed with silica gel. The mixture was eluted with hexane/ethyl acetate (20:80, vol/vol). The purity of the product was confirmed by thin- layer chromatography, where the appearance of a single spot in the isolated sample indicated that the column-separated substance was pure. The spots were visualized by iodine absorption.

Product identification. The isolated product was identified by spectral studies [Fourier transform infrared (FTIR), ^{13}C nuclear magnetic resonance (^{13}C NMR)]. FTIR was recorded on a Nicolet Magna-IR550 (Nicolet, Madison, WI) spectrophotometer. The ^{13}C NMR spectra were recorded on a Bruker DRX-300 (Karlsruhe, Germany) spectrometer at 300 MHz. The chemical shifts are expressed in ppm with tetramethylsilane as internal standard.

Spectra data for DHSA octyl ester. FTIR: 3440 (-OH), 2926 (-C-H), 1741 cm^{-1} (-COO-); ^{13}C NMR: 174.08 (-COO-), 74.47 (-CH-OH at C9), 74.66 (-CH-OH at C10), 64.48 (-O-CH_2-), 34.37–24.96 (-CH_2-), 26.68 (-$\underline{C}H_2CH_3$, alcohol chain), 22.65 (-$\underline{C}H_2CH_3$, acid chain), 14.12 ppm (CH_3).

RESULTS AND DISCUSSION

Screening of enzymes. From a series of experiments which compared several enzymes for their ability to synthesize DHSA-octanol ester, Lipozyme IM and Novozym 435 exhibited the highest activity (data not shown). This result is in agreement with previous work (9,10), although the substrate used was different. Steffen *et al.* (9) produced monoglycerides of (*S*)-17-hydroxy- and 12-hydroxystearic acids at high yields (>80%) with either *R. miehei* or *C. antarctica* lipase as biocatalyst. The formation of wax esters from (*S*)-17-hydroxystearic acid and (*R*)-3-hydroxydecanoic acid with various lipases was carried out by Lang and coworkers (10). They found that the reaction rates were faster when *R. miehei* lipase was the biocatalyst.

Effect of organic solvent type. Reaction mixtures were prepared where organic solvents with different polarities indicated by the log *P* values [the logarithm of partition coefficient of solvent in octanol/water system (11)] were used instead of hexane. Table 1 shows the effect of various organic solvents on esterification of DHSA and 1-octanol using Novozym 435 and Lipozyme IM as catalyst. Both enzymes were active in all organic solvents tested except for dimethylformamide in this particular reaction. The percent conversions of ester were much higher in organic solvent with log *P* values from 2.0 to 4.0. Katchalski-Katzir (12) and Laane *et al.* (12) reported a similar finding on their work on the esterification of tributyrin and heptanol using pancreatic lipase as catalyst. The lower activities of lipase in polar solvents compared with nonpolar solvents could be due to the fact that the more polar solvents could strip the essential water layer around the enzyme, which is important for enzyme catalysis (11,13). However, in the case of solvents with log *P* above 4.0, the percent conversion was relatively lower, as the increased viscosity of the solvents may have decreased the interaction frequency between the substrates and enzyme molecules (13).

Effect of initial a_w. A slight difference in conversion was observed when the substrate, enzyme, and solvent were pre-equilibrated in salt hydrates having a_w from 0.09 to 0.96, (Table 2). This result showed that esterification of DHSA and alcohol was not affected by a_w ranging from 0.09 to 0.96. However, this finding was different from the work by Valivety *et al.* (14). For the reaction between dodecanoic acid and dodecanol, they found that the lipase from *C. antarctica* showed high activity at low a_w whereas lipase from *R. miehei* required a_w of 0.12 for maximal activity.

Effect of alcohol chain length. Figure 1 shows the effect of various fatty alcohol chain lengths on the esterification of DHSA and alcohol. Saturated fatty alcohols with carbon numbers ranging from 8 to 18 were used in this study. The results showed that the time course of the reaction for fatty alcohols of different chain length were quite similar with the

TABLE 1
Effect of Organic Solvent Types on the Esterification of Dihydroxystearic Acid and 1-Octanol[a]

Solvent	Solubility of DHSA[c] (mg/mL)	Log *P*[b]	Conversion (%) Lipozyme IM	Conversion (%) Novozym 435
DMF	0.070 ± 0.001	–0.1	0.0 ± 0.7	3.4 ± 1.0
Acetone	0.00267 ± 0.0003	–0.23	69.2 ± 0.4	75.7 ± 1.3
Diethyl ether	I	0.85	74.4 ± 0.6	77.6 ± 2.7
Chloroform	0.00250 ± 0.0001	2.0	77.6 ± 1.8	79.5 ± 0.2
Toluene	0.00256 ± 0.0002	2.5	80.4 ± 0.5	87.6 ± 0.6
Pentane	I	3.0	81.9 ± 0.6	81.2 ± 1.2
Hexane	I	3.5	80.7 ± 0.4	82.9 ± 1.3
Heptane	I	4.0	79.0 ± 1.0	79.9 ± 0.3
Octane	I	4.5	72.9 ± 1.9	77.5 ± 0.8
Nonane	I	5.1	69.8 ± 1.4	72.0 ± 0.5
Decane	I	5.6	68.1 ± 0.9	67.1 ± 2.6
Dodecane	I	6.6	63.5 ± 0.5	64.6 ± 0.3
Hexadecane	I	8.8	54.1 ± 0.9	51.7 ± 0.7

[a]Reaction mixture contained 2.0 mmol dihydroxystearic acid (DHSA), 4.0 mmol 1-octanol, 3.0 mL organic solvent and 0.3 g immobilized enzyme. Reactions were conducted at 30°C for 5 h. Abbreviations: DMF, dimethylformamide; *P*, partition coefficient; I, insoluble. Both enzymes were from Novo Nordisk A/S (Bagsvaerd, Denmark).
[b]From Laane *et al.* (11).
[c]The solubility test was conducted in triplicate. The amount of DHSA used was 0.1 g. The maximum volume of solvent used was 40 mL. The test was conducted at 30°C under magnetic stirring.

exception of 1-octadecanol (C_{18}-OH). This was probably due to the insolubility of C_{18}-OH at the reaction temperature, 30°C. These results also indicated that there was an effect of alcohol chain length on the initial reaction rates, but the ultimate rates of reaction were more or less the same except for C_{18}-OH. Ghosh and Bhattacharyya (4) reported a similar observation on their work on esterification of 12-hydroxystearic acid and monohydric alcohol.

Effect of the presence of hydroxyl group. The effect of hydroxyl group on esterification was studied using a fatty acid with 0–2 hydroxyl group. Figure 2 shows the changes in percent conversion over reaction time for reactions catalyzed by Lipozyme IM. It indicates that the conversion of stearic acid increased rapidly to a maximum within 1 h whereas it took 3 h for 12-hydroxystearic acid and DHSA. The slower conversion for the one-hydroxyl and two-hydroxyl fatty acid may be attributed to the poor solubility of the compound in the solvent used. Hayes and Kleiman (2) reported that because of the poor solubility, polyol ester-producing reactions in lipophilic organic solvents were slow and that a strategy to increase the rate was to increase the solubility of polyol in the solvent used. This can be done by the employment of derivatized polyol, which is more soluble in lipophilic solvents. The difference in the percent conversion of ester when different types of fatty acids were used may also be due to the fatty acyl substrate selectivity of the lipases.

Effect of substrate mole ratio. The effect of varying the amount of substrate was studied by keeping the amount of DHSA constant at 1.00 and varying the amount of 1-octanol from 0.0 to 5.0 moles. Based on a plot of percent conversion

TABLE 2
Effect of Initial Water Activity (a_w) on the Esterification of DHSA and 1-Octanol[a]

a_w[b]	Conversion (%) Novozym 435	Conversion (%) Lipozyme IM
0.09	79.3 ± 1.7	78.5 ± 0.3
0.12	76.0 ± 1.9	75.5 ± 2.9
0.32	76.1 ± 0.6	79.3 ± 0.2
0.68	74.3 ± 2.7	77.1 ± 1.8
0.86	72.9 ± 0.8	75.6 ± 0.9
0.96	70.2 ± 0.5	72.4 ± 0.6

[a]Reaction mixture contained 2.0 mmol DHSA, 4.0 mmol 1-octanol, 3.0 mL *n*-hexane, and 0.3 g immobilized enzyme. Reactions were conducted at 30°C for 5 h.
[b]Data From Halling (15); reference temperature = 25°C.

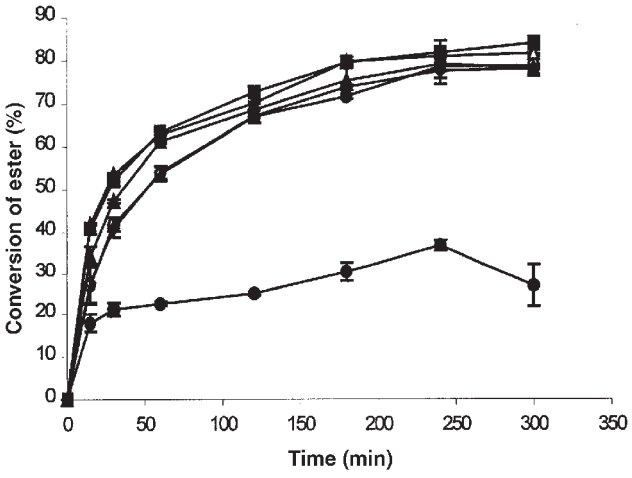

FIG. 1. Percentage esterification of dihydroxystearic acid (DHSA) with different fatty alcohols as a function of time. Reaction conditions: *n*-Hexane (3.0 mL) with 2.0 mmol DHSA, 4.0 mmol alcohol, and 0.3 g Lipozyme IM (Novo Nordisk A/S, Bagsvaerd, Denmark). The reaction mixture was incubated at 30°C. 1-Octanol (Δ), 1-decanol (■), 1-dodecanol (▲), 1-tetradecanol (○), 1-hexadecanol (◇), 1-octadecanol (●).

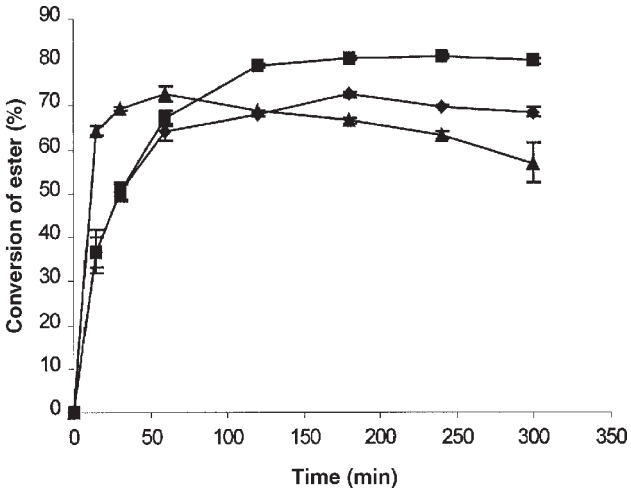

FIG. 2. Effect of the presence of hydroxyl group on the esterification of C_{18} fatty acid and 1-octanol. Reaction conditions: *n*-Hexane (3.0 mL) with 2.0 mmol fatty acid, 4.0 mmol 1-octanol and 0.3g Lipozyme IM. The reaction mixture was incubated at 30°C. Stearic acid (▲), 12-hydroxystearic acid (■), DHSA (◆). For abbreviation and manufacturer see Figure 1.

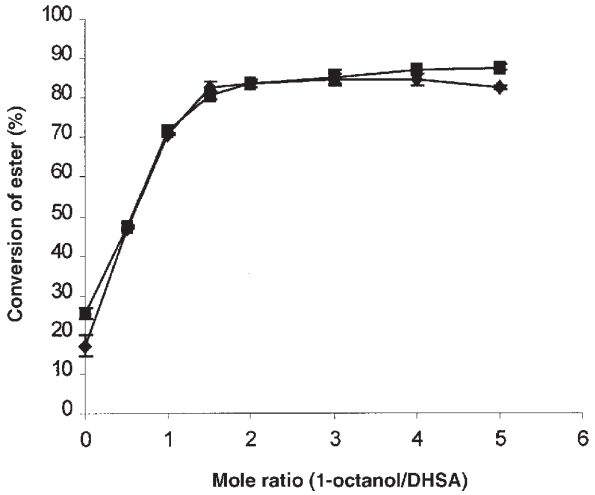

FIG. 3. Effect of the mole ratio of 1-octanol to DHSA on the synthesis of octanoyldihydroxystearate. Reaction conditions: *n*-Hexane (3.0 mL) with 1.0 mmol DHSA, 0.3 g Lipozyme IM or Novozym 435, and varying mmol of 1-octanol. The reaction mixture was incubated at 30°C for 5 h. Lipozyme IM (◆), Novozym 435 (■). For abbreviation see Figure 1. Source of enzymes: Novo Nordisk A/S (Bagvaerd, Denmark).

vs. moles of 1-octanol (Fig. 3), Lipozyme IM and Novozym 435 exhibited an increasing trend in percent conversion with increasing amount of 1-octanol from 0.0 to 2.0 mole. However, further increase of alcohol did not have any effect on percent conversion. In this case, DHSA substrate limitation may be the reason. Therefore, the mole ratio of alcohol to acid of 1.0–2.0 was sufficient for this esterification reaction. This result also indicated that Lipozyme IM and Novozym 435 were not inhibited by the excess alcohol substrate.

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