Lipase-Catalyzed Synthesis of Fatty Acid Diethanolamides

Kuan Ju Liu,[†] Ahindra Nag,[‡] and Jei-Fu Shaw*[,‡]

Department of Food Science and Technology, Tungfang Junior College of Technology and Commerce, Kaohsiung, Taiwan, 82901, and Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan 11529

Diethanolamides are nonionic emulsifiers widely used in industries such as cosmetics and as corrosion inhibitors. *Candida antarctica* lipase (Novozym 435) was used to catalyze the amidation of various fatty acids with diethanolamine. Contents of fatty acids, metal ions, and water affected the yields of diethanolamides. Hexanoic acid was the best substrate among all acyl donors. Yields of hexanoyl diethanolamide (HADEA), lauroyl diethanolamide (LADEA), and oleoyl diethanolamide (OADEA), obtained after 24 h of lipase-catalyzed reaction at 50 °C and 250 rpm with 90 mM fatty acid and 360 mM diethanolamine in acetonitrile, were 76.5, 49.5, and 12.1%, respectively. Addition of 1 mM metal salts increased the yields of HADEA and LADEA. Kinetic analysis showed that the yields of HADEA and LADEA in lipase-catalyzed reactions were largely associated with the rate of the forward reaction constant k_1. Anhydrous enzyme was found to be the best for the amidation reaction. Study on the enzyme operational stability showed that *C. antarctica* lipase retained 95 and 85% of the initial activity for the syntheses of HADEA and LADEA, respectively (even after repeated use for 10 days). The reaction runs smoothly without the use of hazardous reactants, and the developed method is useful for the industrial application.

Keywords: *Fatty acids; lipase; Candida antarctica; diethanolamides*

INTRODUCTION

Fatty acid amides have a broad spectrum of use as detergents, shampoos, cosmetics, lubricants, foam control agents, fungicides, corrosion inhibitors, and water repellents (*1*). Owing to their low reactivity and thermal properties they are used for the preparation of anti-slip and anti-block additives in polyethylene films and as flow improvers (*2*).

The desired amide can be produced by a Schotten Bauman reaction using fatty acyl chloride and amine as reactants. Jorden and Port reported conversion of *n*-butylamine and methyl stearate to *n*-butyl stearamide using sodium methoxide as catalyst (*3*). Magg (*4*) suggested the preparation of desired diethanolamide and amine ester by reacting 2 mol of fatty acid and 1 mol of diethanolamine at 180 °C. However, such methods are rather hazardous.

Diethanolamides can be also prepared by amidation of natural oil. Bilyk et al. (*5*) synthesized monosubstituted fatty amides by reacting primary amine with vegetable oil, tallow, and fish oil. The reaction was carried out at the boiling point of amine using a molar ratio of oil and amine of 1:8. Amides are produced industrially from the fatty acids and alkanolamines by heating them at 140−160 °C during 6−14 h in an agitated vessel with a means of removing excess of amine, water, or alcohol (*6*). Another industrial method is based on the reaction of 2 mol of fatty acid with 1 mol of ethylenediamine at 180−185 °C during 6 h under nitrogen and with continuous removal of water (*7*). However, high temperature causes self-condensation of diethylamine that results to form *N*,*N*′-bis(2-hydroxyethyl)piperazine or morpholine (*8*, *9*). Thus, all of these processes are tedious and require large amounts of energy.

In view of these drawbacks, an attempt was made to develop an enzymatic reaction as an alternative low-cost and low-energy-consuming industrial process. In the present work, we found that various diethanolamides can be efficiently synthesized from diethanolamine and various fatty acids using *Candida antarctica* lipase (Novozym 435). The effects of acyl donors, organic solvents, temperature, water content, kinds of lipases, and operational stability were investigated.

MATERIALS AND METHODS

Nine lipases were obtained from available commercial sources. Lipase from *Aspergillus niger* (Amano AP6), *Mucor* sp. (Amano MAP-10), *Penicillium camembertii* (Amano G), *Pseudomonas cepaceia* (Amano PS), and *Rhizopus* sp. (Amano N, concentrated) were purchased from Amano International Enzyme Co. (Nagoya, Japan). Lipases from *Candida antarctica* (Novozym 435) and *Mucor miehei* (Lipozyme IM) were purchased from Novo Nordisk Inc. (Danbury, CT). Porcine pancreatic lipase and lipase from *Candida cylindracea* were purchased from Sigma Chemical Co. (St. Louis, MO). Diethanolamine, hexanoic acid, hexanoic anhydride, lauric acid, lauric anhydride, oleic acid, oleic anhydride, methyl laurate, and tricaprin were purchased from Merck Chemical Co. (Darmstadt, Germany). All other chemicals were of reagent grade.

For a standard reaction, the commercial lipase powder (0.15−0.25 g) was added to a reaction mixture (1 mL) containing 90 mM fatty acid and 360 mM diethanolamine in acetonitrile. The reaction mixture was incubated in an orbital shaker at 250 rpm and 50 °C during 24 h. At various time points 1 μL of the reaction mixture was withdrawn and analyzed.

The preliminary identification of the products was done according to Fink's method (*10*). Analytical thin-layer chro-

* Corresponding author (fax 886-2-27821605; e-mail boplshaw@gate.sinica.edu.tw).

[†] Tungfang Junior College of Technology and Commerce.

[‡] Academia Sinica.

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Table 1. Diethanolamide Formation by Lipases Derived from Different Sources

source	trade name or brand	yield[a] (%)			hydrolytic activity[b] (units/g)
		HADEA	LADEA	OADEA	
Aspergillus niger	Amano AP-6	0.0	0.0	0.0	908.6
Candida cylindracea, type VII	Sigma	0.0	0.0	0.0	762.8
Candida antarctica	Novozym 435	49.2	45.8	11.7	14.5
Mucor sp.	Amano MAP-10	0.0	0.8	0.0	45.7
Mucor miehei	Lipozyme IM	0.0	1.0	0.0	7.1
Penicillium camembertii	Amano G	1.1	0.3	0.1	479.1
Pseudomonas cepacia	Amano PS	0.3	0.8	0.0	925.9
Rhizopus sp.	Amano N, concentrated	0.0	0.0	0.0	71.9
porcine pancreatic lipase	Sigma	3.9	8.4	0.4	14.2

[a] Lipase (0.15 g) was added to a reaction mixture (1 mL) containing 360 mM diethanolamine and 90 mM appropriate fatty acid. The reaction was carried out during 24 h in acetonitrile at 40 °C. [b] The lipase hydrolytic activity was measured by *p*-nitrophenyl butyrate as substrate. One unit of enzyme was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute.

matography (TLC) was performed on silica gel 60 F_{254}-coated glass plates. The TLC plate was spotted with 10 μL of analyzed products. The plate was then developed with mixture (47.6:47.6:4.8) of chloroform/toluene/methanol (v/v/v). Upon drying, the plate was sprayed with 50% sulfuric acid solution that permitted detection of the amides by color spots. The amide compounds also were purified with column chromatography using a solvent mixture (49.3:49.2:1.5) of chloroform/toluene/methanol (v/v/v). Synthetic standards of corresponding amides were prepared according to the method of Bestline et al. (*9*).

The identity of diethanolamides obtained with lipase was proved using standard methods. Infrared (IR) spectra were recorded on a Bomen MB-100FT spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker nuclear magnetic resonance spectrometer AMX-500. Chemical shifts are expressed relative to tetramethylsilane. High-resolution mass spectra were recorded on GC-MS spectrometer HP5890 GC/VG70-250S MS. Elemental analysis were performed on a Fisons EA 1108 apparatus. Melting points were determined using a Buchi 535 apparatus.

Lauroyl diethanolamide (LADEA) possessed the following characteristics: mp, 38 ± 0. 5 °C; nitrogen content, 4.80 (calculated 4.87); IR 2990, 2352, 1616, 1449, 1061 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.42 (bs, 2H), 3.63 (m, 4H,) 3.38 (m, 4H), 2.57 (m, 1H), 2.30 (t, J = 6.5 Hz, 3H), 1.49 (m, 2H), 1.35−1.06 (m, 16H), 0.78 (t, J = 6 Hz, 3H); ^{13}C (75 MHz, DMSO-d_6) δ 186.0 (C), 60.6 (CH_2), 60.3 (CH_2), 51.9 (CH_2), 33.4 (CH_2), 31.7 (CH_2), 29.4 (six CH_2 signals overlapped), 25.6 (CH_2), 22.5 (CH_2), 13.9 (CH_3); GC-MS (EI), *m/e* 289 (M^+), 288 ($M^+ - 1$), 244 ($M^+ - CH_2 - CH_2 - OH$).

The kinetic parameters of reaction, namely, the forward rate constant k_1 and the backward rate constant k_{-1}, were evaluated according to the method described by Levenspiel (*15*).

The effect of water content on the lipase-catalyzed synthesis of diethenoamides was studied. The lipase was lyophilized on a Savant Speed Vac concentrator (Savant Instruments, Inc., Farmingdale, NY) under 50 mTorr during 24 h. Water was removed from organic media by a 3 Å molecular sieve (Merck). The lipase hydrolytic activity was measured according to the procedure described by Rúa et al. (*11*).

RESULTS AND DISCUSSION

Among nine commercial lipases tested *C. antarctica* lipase showed the best catalytic efficiency and specificity (Table 1) for enzymatic synthesis of hexanoyl diethanolamide (HADEA), lauroyl diethanolamide (LADEA), and oleoyl diethanolamide (OADEA). Identity of diethanolamides obtained with lipase was proved using standard methods.

The yields of HADEA, LADEA, and OADEA, obtained after 24 h of *C. antarctica* lipase-catalyzed reaction at 40 °C and 250 rpm with 90 mM fatty acid and 360 mM diethanolamine, were 49.2, 45.8, and 11.7%, respectively (Table 1). Other enzymes were of comparable catalytic activity. The specific activities of each lipase were measured by the hydrolysis of *p*-nitrophenyl butyrate and are listed as a reference for the comparison of lipase activity units. The comparison of various enzymes (Table 1) demonstrates that the better hydrolytic catalysts (on *p*-nitrophenyl butyrate) are not necessarily best for "dehydration" to achieve the amides.

Table 2. Effect of Acyl Donors on the Lipase-Catalyzed Synthesis of Diethanolamides

diethanolamide	acyl donor	yield[a] (%)
HADEA	hexanoic acid	76.7
	hexanoic anhydride	72.3
	methyl hexanoate	58.0
	tricaprin	18.2
LADEA	methyl laurate	56.5
	lauric acid	49.5
	lauric anhydride	35.8
	trilaurin	31.1

[a] Lipase (0.2 g) was added to a reaction mixture (1 mL) containing 360 mM diethanolamine and 90 mM appropriate acyl donor. The reaction was carried out during 24 h in acetonitrile at 50 °C.

Table 3. Kinetic Constants for Lipase-Catalyzed Reactions of Various Fatty Acids with Diethanolamine[a]

fatty acid	carbon no.	k_1	k_{-1}	k_{-1}/k_1
hexanoic acid	$C_{6:0}$	0.4	0.5	1.3
lauric acid	$C_{12:0}$	0.1	0.2	2.0
myristic acid	$C_{14:0}$	0.03	0.1	3.3
palmitic acid	$C_{16:0}$	0.01	6.1	610.0
stearic scid	$C_{18:0}$	0.0003	1.1	3666.7
oleic acid	$C_{18:1}$	0.0006	1.1	1833.3

[a] Lipase (0.25 g) was added to a reaction mixture (2 mL) containing 360 mM diethanolamine and 90 mM fatty acid. The reaction was carried out during 24 h in acetonitrile at 50 °C.

The acyl donors greatly affect the *C. antarctica* lipase-catalyzed synthesis of diethanolamides (Table 2). Trilaurin and tricaprin were found to be poor substrates, whereas hexanoic acid and methyl laurate were the best ones for the synthesis of HADEA and LADEA, respectively.

The effects of fatty acid chain length on the synthesis of diethanolamide are shown in Table 3. *C. antarctica* lipase appeared to favor short-chain fatty acids as the substrates. Namely, hexanoic acid was the most suitable substrate for the reaction. In this case, the forward rate constant k_1 demonstrated the lowest value that decreased k_{-1}/k_1 ratios and increased the selective accumulation of amide (Table 3).

Table 4 shows the temperature effect on *C. antarctica* lipase catalytic activity. Previously, Nag et al. (*13*) have shown that high temperature changes the conformation of enzymes that results to the free energy change in the

Table 4. Lipase-Catalyzed Amidation of Hexanoic, Lauric, and Oleic Acids at Various Temperatures

	yield of diethanolamide[a] (%)		
temp (°C)	HADEA	LADEA	OADEA
10	2.8	0.6	0.0
20	26.5	13.4	0.7
30	44.6	27.3	1.7
40	49.2	45.8	11.7
50	76.5	49.7	12.1
60	81.5	52.9	23.0

[a] Lipase (0.15 g) was added to a reaction mixture (1 mL) containing 360 mM diethanolamine and 90 mM fatty acids in acetonitrile at various temperatures for 24 h.

Table 5. Effect of Organic Solvents on the Lipase-Catalyzed Amidation of Haxanoic, Lauric, and Oleic Acids at 50 °C

	yield[a] (%)		
solvent	HADEA	LADEA	OADEA
acetone	11.4	5.8	0.0
acitonitrile	72.3	31.1	0.7
chloroform	41.2	22.7	0.1
diethyl ether	12.7	32.5	2.4
ethyl acetate	0.4	3.1	0.0
tert-buyl alcohol	55.4	27.6	0.4
tetrahydrofuran	72.9	32.6	0.5
toluene	10.4	26.7	0.2

[a] Lipase (0.125 g) was added to a reaction mixture (1 mL) containing 360 mM diethanolamine and 90 mM fatty acids. The reaction was carried out during 24 h in various organic solvents at 50 °C.

system, which might affect substrate binding capacity and therefore affect the yield of reaction. Because *C. antarctica* lipase is quite thermostable, running the reaction at 40 °C or higher up to the point (that was above 60 °C) allowed yields of 81.5, 52.9, and 23.0% to be obtained for the corresponding HADEA, LADEA, and OADEA. However, the yields for HADEA and LADEA at 50 °C were close to those obtained at 60 °C. Thus, the reaction temperature of 50 °C was chosen as an optimal temperature for further reactions to save energy and avoid possible enzyme denaturation during long operation for the industrial applications.

Generally, organic solvents greatly affect the yield of *C. antarctica* lipase-catalyzed diethanolamides. Table 5 shows that the higher yield of HADEA and LADEA can be obtained in the presence of acetonitrile and tetrahydrofuran, which are hydrophilic organic solvents. However, the yield of OADEA was not satisfactory in any organic solvents.

It has been proposed that organic solvents with high log *P* values (the logarithm of the partition coefficient in a standard octanol−water two-phase system) are the most suitable for biocatalysis in organic media (*14*). We disclosed that the optimum log *P* for the synthesis of propylene glycol monostearate by *Pseudonmonas* lipase (Amano PS) is between 2.9 and 3.5 (*15*). The most suitable organic media for the synthesis of propylene glycol monoesters of eicosapentanoic acid and docosahexaenoic acid by *M. miehei* lipase (Lipozyme IM20) are *n*-hexane/*tert*-butyl alcohol (9:1) mixed organic solvents (*16*). Our present experiments suggest that the best organic solvents for the synthesis of diethanolamides by *C. antarctica* lipase are hydrophilic organic solvents (namely, acetonitrile and tetrahydrofuran). Thus, the hypothesis that hydrophobic organic solvents with high log *P* are the most acceptable for biocatalysis cannot be universally applicable.

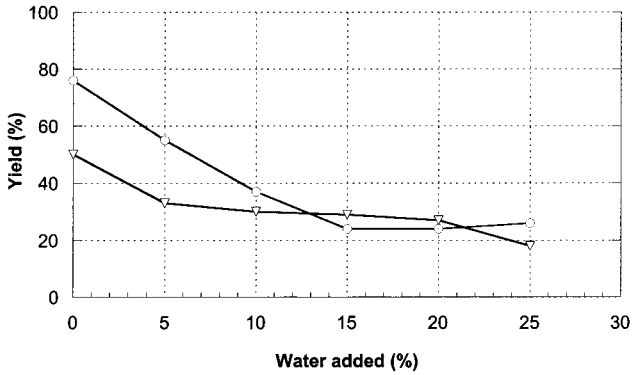

Figure 1. Effect of water on the synthesis of HADEA (open circles) and LADEA (open triangles) by Novozyme 435. The lipase (0.15 g) was added to a reaction mixture (1 mL) containing 90 mM fatty acid and 360 mM diethanolamine in acetonitrile with various amounts of added water at 50 °C during 24 h.

Table 6. Effect of Diethanolamine/Fatty Acid Ratio on the Lipase-Catalyzed Synthesis of Diethanolamide

diethanolamine/ fatty acid ratio	yield[a] (%)	
	HADEA	LADEA
0.1	0.2	0.1
0.3	1.8	0.8
0.5	10.2	4.5
1.0	30.1	15.3
2.0	57.4	27.4
4.0	76.5	51.3
10.0	51.6	2.2

[a] Lipase (0.15 g) was added to a reaction mixture (1 mL) containing various ratios of diethanolamine/fatty acid. The reaction was carried out in acetonitrile during 24 h at 50 °C.

Table 7. Effect of Various Metal Salts on the Lipase-Catalyzed Synthesis of Diethanolamides

	yield[a] (%)	
metal salt	HADEA	LADEA
none	76.5	51.3
$CaCl_2$	90.3	76.0
$CuCl_2$	92.2	70.7
$MgCl_2$	86.7	59.8
$MnCl_2$	79.2	67.7
$SrCl_2$	88.1	63.1
$ZnCl_2$	99.8	70.8

[a] Lipase (0.15 g) was added to a reaction mixture (1 mL) containing 360 mM diethanolamine, 90 mM fatty acid, and 1 mM various metal salts. The reaction was carried out during 24 h in acetonitrile at 50 °C.

Figure 1 shows effect of water on the synthesis of HADEA and LADEA. Apparently, lipase performance is highest under anhydrous conditions for amidation with either hexanoic or lauric acid. As the reaction proceeded with elimination of water (*4*), further addition of water might favor the hydrolysis of diethanolamide.

The effect of the diethanolamine/fatty acid ratio on the lipase-catalyzed amidation is shown in Table 6. Maximum yields of both HADEA and LADEA were achieved at the ratio of 4. Higher ratios decreased the yield of the products, which is probably due to the increased formation of monoesters and diesters of diethanolamine or diethanolamide.

The effect of metal ions on the rate of amidation was studied. Addition of 1 mM divalent salts significantly increased the yield of the amides (Table 7). Production of different amides requires the different metal ions to improve the yield. Namely, $ZnCl_2$ remarkably increased

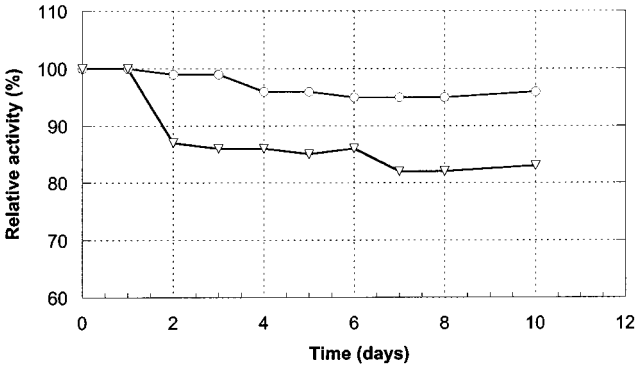

Figure 2. Operational stability of Novozyme 435. The reaction mixture was refreshed every day, and relative activity was assayed immediately after each change. The lipase (0.15 g) was added to a reaction mixture (1 mL) containing 90 mM fatty acid and 360 mM diethanolamine at 50 °C and 250 rpm during 24 h. The open circles and the open triangles represent HADEA and LADEA, respectively.

the yield of HADEA to 99.8%, and $CaCl_2$ greatly increased the yield of LADEA to 76%.

Figure 2 represents the operational stability of lipase for amidation of diethanolamine with lauric and hexanoic acid. After 10 days of repeated use at 50 °C the lipase retained 95 and 85% of its initial activity for the synthesis of HADEA and LADEA, respectively. Such stability of *C. antarctica* lipase makes the enzyme economically feasible for industrial production of the amides.

ABBREVIATIONS USED

HADEA, hexanoyl diethanolamide; LADEA, lauroyl diethanolamide; OADEA, oleoyl diethanolamide.

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