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Enzymatic Chemoselective Synthesis of Secondary-Amide Surfactant from *N*-Methylethanol Amine

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Efficient selective synthesis of the secondary amide surfactant *N*-methyl lauroylethanolamide from methyl laurate and *N*-methylethanol amine by carrier-fixed Chirazyme L-2 (*Candida antarctica*) using a kinetic strategy has been demonstrated. When different solvents were screened for product yields using Chirazyme L-2, acetonitrile was found to be optimal. The rate of the reaction increased sharply by increasing the molar ratio of the reactants and the reaction temperature. When the reaction was performed at 50°C for 36 h with 50 mmol ester and 100 mmol amine, the product was obtained in a 97.1% yield. With 50 mmol ester and 150 mmol amine, the highest yield (97.3%) was obtained after 16 h of incubation at 50°C. It took only 5 h to get a yield of 95.8% at 60°C using 50 mmol ester and 200 mmol amine. The enzyme activity in the amidation reaction mixture did not decrease notably even after six uses.

[**Key words:** Chirazyme L-2, *N*-methyl lauroylethanolamide, secondary amide surfactant, methyl laurate, *N*-methylethanol amine]

Fatty acid amides are of considerable interest and economic importance and have therefore been the object of much research and industrial attention. They are now produced on a large scale as they are useful as fiber lubricants, detergents, flotation agents, textile softeners, antistatic agents, wax additives, and plasticizers. The amides of lauric acid are widely applied in shampoos, bathing lotions, dermal cleaning agents and skin creams (1). Fatty acid amides are semicommodities that are produced from the fatty acids by reaction with anhydrous ammonia at approximately 200°C and 345–690 kPa (2). Owing to their heat sensitivity, the manufacture of these amides requires additional distillation steps in order to meet purity specifications. Hence, a low temperature synthesis that circumvents the need for additional purification is potentially attractive.

So far, chemical approaches have only been utilized for their mass production. At the same time, some lipases have successfully been used for the synthesis of primary amides of oleic and palmitic acids (2; Mori, N., Uemura, S., and Iwasaki, R., Japanese open patent 153985, Jun. 22, 1993). Lipase-catalyzed ammoniolysis of amino acid esters, carboxylic acids and triglycerides has been described as an attractive method to obtain the corresponding amides (3–5). The formation of *N*-lauroyl-β-alanine ethyl ester and 3-(*N*-lauroylamino)-propionitrile using immobilized lipase (6), and synthesis of *N*-lauroyl-β-amino propionitrile using a packed-bed reactor have been reported (7). Enzymatic synthesis of hydroxyamides from *N*-methylglucamine (8, 9) and ethanolamine has also been reported (10). The enzymatic synthesis of secondary amides has been unsuccessful with a few exceptions (6), in contrast to that for primary amides (11). Here, we describe our results from a study of enzyme-catalyzed chemoselective synthesis of secondary amide. We used Chirazyme L-2 to catalyze a selective reaction of methyl laurate with *N*-methyl ethanolamine, which contains one hydroxyl and one amine group (Fig. 1).

MATERIALS AND METHODS

Reagents Methyl laurate and *N*-methylethanol amine were gifts from Kawaken Chemicals, Fukui. All other chemicals were from commercial sources and were used without further purification.

Enzymes Lipases from *Pseudomonas cepacia* (PS), *Pseudomonas fluorescens* (AK), *Candida rugosa* (AY), *Aspergillus niger* (A), *Rhizopus* sp. (F-AP15), *Mucor javanicus* (M), *Geotricum candidum* (GC-20), *Rhizopus* sp. (D), *Penicillium roqueforti* (R), *Aspergillus* sp. (PZ-6), *Humicola langinosa* (CE), *Penicillium aurantiogriseum* (G), *Geotricum candidum* (GC-20), *Rhizopus niveus* (Neolase F), porcine pancrease (Pancreatin F), protease from *Bacillus subtilis* (N), and esterase (AC 409) were gifts from Amano Pharmaceutical (Nagoya). Lipases from *Rhizomucor miehei* (Lipozyme), *Aspergillus niger*, *Candida lipolytica*, *Mucor javanicus* and *Rhizopus arrhizus* were purchased from Fluka Chemie AG (Buchs, Switzerland); lipases from *Candida rugosa*, pig liver (PLE), wheat germ, and Type II lipase and Pancreatin were purchased from Sigma Chemical Company (St. Louis, MO, USA); lipases from *Pseudomanas aeruginosa* (Lipoprotein lipase) and *Pseudomonas* sp. (Toyozyme LIP) were purchased from Toyo Jozo (Shizuoka); lipase from *Rhizopus niveus* was purchased from

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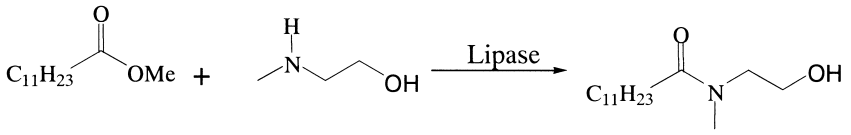

Methyl laurate *N*-Methylethanol amine *N*-Methyllauroylethanolamide

FIG. 1. Enzymatic amidation in organic solvents.

Kanto Chemicals (Tokyo); lipase from *Rhizopus delemer* was purchased from Seikagaku Kogyo (Tokyo) and Chirazyme (L-2) was purchased from Boehringer Mannheim (Mannheim, Germany). The specific activity of Chirazyme (L-2) is 10,000 propyl laurate units (PLU) /g and one PLU is defined as the amount of enzyme that catalyzes the formation of 1 μmol of ester from propyl alcohol and lauric acid per min.

Screening of enzymes for amide synthesis In 1.5 ml Eppendorf tubes, methyl laurate (50 mmol), *N*-methylethanol amine (100 mmol) dissolved in 1 ml acetonitrile and 2 mg of enzyme were added. The reaction mixtures were shaken at 30°C for 20 h and product formation was checked by TLC with the solvents chloroform and methanol in a 10:1 ratio (v/v) as a mobile phase and visualization of spots was achieved by spraying phosphomolybdic acid-sulphuric acid or ninhydrin solution, followed by heating the plates at 120°C. Percent molar yields were determined by HPLC with an ODS column (Cosmosil $5C_{18}$; Nacalai Tesque, Kyoto; mobile phase, CH_3CN/0.02 M 1-hexanesulphonic acid sodium salt (H_2O)=60/40; flow rate, 0.7 ml/min; retention time for *N*-methyl lauroyletanolamide, 12.9 min), which was monitored by a refractive index detector (Shodex RI-101; Showa Denko, Kanagawa). Infrared (IR) spectra of a sample in the KBr pellet were recorded from 400 to 4600 cm^{-1} using a Shimadzu FTIR-8100 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL LA-400 spectrophotometer (JEOL, Tokyo) for solutions in CD_3OD with tetramethylsilane as an internal standard and *J* values are given in hertz (Hz).

The IR spectra of *N*-methyl lauryoleathanolamide: (OH)=3400 cm^{-1}, CH=2800–2900 cm^{-1}, and (CO-N)=1651 cm^{-1}. ^1H NMR δ; (MeOD, 400 MHz); 0.96 (vt, *J*=7.2 Hz, 3H), 1.2–1.3 (br, 18H), 1.58 (brs, 2H), 2.2 (t, *J*=7.0 Hz, 2H), 2.3 (t, *J*=7.0 Hz, 1H), 2.90 (s, 3H), 3.42 (m, 1H), 3.5 (m, 1H). ^{13}C NMR δ (MeOD, 100 MHz); 14.1, 22.6, 26.0, 28.5, 29.0, 29.4, 29.5, 29.6, 29.7, 32.0, 34.3, 35.2, 52.4, 58.7, 172.3.

RESULTS AND DISCUSSION

Mild conditions, a minimal need for protective groups, a lack of by-products, high regio- and enantioselectivity, and low synthesis costs make the enzymatic syntheses of amides favorable compared with chemical syntheses (12). To date, proteases such as thermolysin and subtilisin are particularly utilized for large-scale amide production (13, 14). However, these enzymes are known to be specific for certain amino acids and are quite sensitive to inactivation by organic solvents. Among the hydrolases, lipases are promising catalysts for the synthesis of peptides, polymers, surfactants and new detergents at low cost, as they have been proven to catalyze the formation of amide bonds in organic solvents (15–20). Two strategies for the formation of amide bonds can be considered: thermodynamic control and kinetic control (12). In the thermodynamic approach, the equilibrium is shifted toward the synthesis instead of the hydrolysis and this could be achieved by changing the reaction conditions. For example, an increase in the concentrations of the starting materials or the precipitation of the product will favor the amidation reaction, with the replacement of the water molecule with an organic solvent. In kinetically controlled reactions, the starting material is an activated carboxyl compound such as an ester. The ester is activated by the enzyme through an acyl-enzyme intermediate, which can be further attacked by an amine or a water molecule. Because the kinetically controlled syntheses require acyl-enzyme intermediates, only serine or thiol hydrolases, such as lipases, subtilisins and papain, can be used. Metalloproteases such as thermolysin are suitable only in thermodynamically controlled reactions.

We designed a kinetically controlled reaction of methyl laureate with *N*-methyl ethanolamine using commercially available and donated enzymes. After screening the solvents, we selected acetonitrile as the most suitable solvent for the reaction, because of its capability to dissolve both of the reactants without affecting the enzymes. Thirty-two enzymes were screened for their product forming ability using TLC. Three lipases, namely, Chirazyme L-2 from *Candida antarctica*, Toyozyme, and Amano PS, were rested positive in this assay and their yields obtained using these enzymes were estimated by HPLC. The highest yield of 51.4% was observed with Chirazyme, followed by Toyozyme and Amano PS (Table 1).

The reaction mixture with Chirazyme predominantly gave two detectable spots by TLC using phosphomolybdic acid-sulphuric acid visualization (R_f: 0.25 and 0.45). The product at R_f=0.45 was purified by silica gel column chromatography from a reaction scaled up 100-fold compared with that of the screening reaction using Chirazyme and its structure was confirmed using IR and NMR spectra, as shown in the Materials and Methods section. The product detected at R_f= 0.25 by TLC was ninhydrin positive and produced a peak at a retention time of 8.3 min in the HPLC analysis. The ratio of the peak areas at retention times of 8.3 and 12.9 min was roughly 2:3. Although we attempted to isolate the product, not only by silica gel column chromatography, but also by physically scraping the spot from the thin layer chromatograph, the ninhydrin positive product could not be isolated and we found only a few decomposed products and *N*-methyl lauryoleathanolamide on the thin layer chromatograph, as described below, after evaporation. When Amano PS was

TABLE 1. *N*-Methyl lauroylethanolamide yield catalyzed by several enzymes

Enzyme	Yield (%)
Chirazyme	51.4
Toyozyme LIP	30.2
Amano PS	9.6

With lipase CE, lipase type II, pancreatin and pancreatin F, the yield was less than 1%.

TABLE 2. Screening of solvents for amide synthesis using Chirazyme

Solvent	Yield (%, by HPLC)
Acetonitrile	51.5
n-Hexane	18.4
Toluene	2.6
Diisopropylether	1.4
Methylene chloride	1.3

The reactions were monitored by both TLC and HPLC. In *t*-butylmethylether, 1,4-dioxane and 4-methyl-2-pentanone, the yield was less than 1%. No product was detected in methanol, iso-propanol, 1-butanol, 2-butanol, 2-methoxyethanol, acetone, cyclohexane, 2-butanone, THF, pyridine, or H_2O.

TABLE 3. Effect of molar ratio of reactants, temperature and reaction time on *N*-methyllauroylethanolamide yield

Run	Ester (mmol)	Amine (mmol)	Reaction time (h)	Temperature (°C)	Yield (%)
1	50	100	24	30	58.8
2	50	100	60	30	77.3
3	50	100	40	40	84.7
4	50	100	36	50	97.1
5	50	100	30	60	96.2
6	50	150	24	30	77.3
7	50	150	36	30	95.6
8	50	150	20	40	93.9
9	50	150	16	50	97.3
10	50	150	12	60	96.6
11	50	200	24	30	96.2
12	50	200	24	30	96.2
13	50	200	16	40	93.4
14	50	200	8	50	95.4

used, the ninhydrin-positive spot was also detected at R_f= 0.25 by TLC. The main product formed with Amano PS was also isolated by a similar procedure and was confirmed as *N*-methyl lauryoleathanolamide by NMR and IR spectra.

When different solvents were tested for their product forming ability using Chirazyme by TLC and HPLC, acetonitrile was found to be the best solvent with 51.5% yield, followed by *n*-hexane with an 18.4% yield (Table 2).

The main product in *n*-hexane was isolated by a similar procedure as described above, and was similarly confirmed as *N*-methyl lauryoleathanolamide by NMR and IR spectra. A spot of *N*-methyl lauryoleathanolamide together with a trace of the ninhydrin positive spot was also detected when the reaction occurred in *n*-hexane and was catalyzed by Chirazyme. The products formed in toluene, diisopropylether, methylene chloride, *t*-butylmethylether, 1,4-dioxane and 4-methyl-2-pentanone all gave the same retention time (12.9 min) as that in *N*-methyl lauryoleathanolamide, by HPLC. To optimize the amount of biocatalyst, different amounts of Chirazyme ranging from 2 mg to 10 mg, were used to perform the reaction at 30°C for 24 h. Yields were 58.8% and 58% for 2 mg and 5 mg of enzyme and the yields further decreased with further increases in the amount of enzyme, that is, 53.2% for 7 mg of enzyme and 50.8% for 10 mg. In all further experiments, 2 mg of enzyme was used.

Effect of molar ratio of reactants, temperature and reaction time on *N*-methyl lauroylethanolamide synthesis Reactions were carried out at 30°C to 60°C for 8 to 24 h using different molar ratios of methyl laurate and *N*-methylethanol amine. The yield of the product was increased when an excess of amine was employed as shown in Table 3.

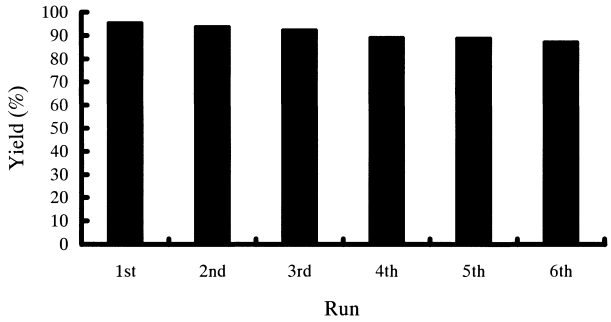

FIG. 2. Repeated use of Chirazyme for enzymatic amidation of *N*-methylethanolamine in methyl laurate.

The highest yield of 97.3% was obtained when a threefold excess of amine (ester 50 mmol, amine 150 mmol) was used. When the reaction was performed with a twofold excess of amine (ester 50 mmol, amine 100 mmol), improvements in the yield were observed by not only prolonging the incubation time from 24 to 60 h (58.8% and 77.3% yields in runs 1 and 2, respectively), but also by raising the temperature to 50°C and incubating for 36 h (97.1% yield in run 4). No further improvement was observed by increasing the temperature above 50°C (runs 2–5). Further reactions were performed to determine the optimum time to achieve the maximum yield for each temperature and molar ratio. With increases in the molar ratio (1:2, 1:3, and 1:4), higher yields were obtained at 30°C in a 24-h reaction (58.8%, 77.3%, and 96.2% yields in runs 1, 6, and 11, respectively). However, significant improvements in the yields were observed when the reaction temperatures were shifted from 30°C to 50°C (77.3% to 97.1% yields in runs 2–5; 95.6% to 97.3% yields in runs 7–10, respectively) with shorter incubation times. Summing up, by considering that the use of less amine is favorable, one of the optimum yields (97.1%) was obtained when the reaction was performed at 50°C for 36 h with an ester to amine ratio of 1:2 (run 4). When a shorter reaction time is necessary, a reaction can be performed at 50°C for 16 h with an ester to amine ratio of 1:3 (run 9 in a 97.3% yield), or at 60°C for 5 h with an ester to amine ratio of 1:4 (1st run of Fig. 2 in 95.8% yield). The difference in product yield at different ratios of the precursor reagents is essentially governed by variations in the pH of the reaction mixture and reflects changes in the acid-base properties of the precursor reagents.

Repeated use of enzyme For the repeated use of the enzyme, reactions were carried out with a fourfold excess of amine (50 mmol of ester, 200 mmol of amine) with 2 mg of Chirazyme at 60°C for 5 h. The enzyme Chirazyme was filtered from the reaction mixture and was used for the next reaction cycle. As shown in Fig. 2, the enzyme did not lose its catalytic activity at 60°C and could be used six times (runs 1–6, in 95.8%, 94.2%, 92.8%, 89.6%, 89.2%, and 97.7% yields, respectively) without any treatment between runs and with little loss of activity. In the presence of an excess of amine, the selectivity for the desired amide increased markedly during the enzymatic synthesis of amide

surfactant from diethanolamide (10).

There are a number of reports on the enzymatic synthesis of biosurfactants with a primary amide bond, but only a few examples of the enzymatic synthesis of a secondary amide are available. In the case of secondary amide synthesis, the hydroxyl group of *N*-methylethanol amine can also react with a fatty acid ester; thus, it is necessary to find suitable conditions allowing the selective acylation of amine functionality. Izumi *et al*. have described the synthesis of amides from fatty acid esters and primary amines using Chirazyme L-2 (*Candida antartica* lipase) (6), although the conversion of a secondary amine, *N*-methylglycine ethyl ester to methyl laurate in THF or chloroform was unsuccessful. We have determined the reaction conditions for secondary amide synthesis and the yield was nearly quantitative. The enzymatic *N*-acylation of *N*-methyl-glucamine in hexane using lipase from *Rhizomucor miehei* has been demonstrated. *N*-Methyl-glucamine is solubilized by free oleic acid addition, resulting in the formation of an ion pair between the acid and the amine, which seems essential for amide synthesis (9). In addition, the synthesis of glucamide surfactants has been carried out by acylation of *N*-methyl-glucamine in the presence of Chirazyme L-2, in the polar protic solvent 2-methyl-2-butanol (21). Because the substrate *N*-methyl-glucamine has both amine and hydroxyl groups, the reaction with methyl oleate catalyzed by Chirazyme gave a mixture of amide and mono-ester, with a higher preference for amide formation. Similarly, in this study, the minor ninhydrin positive compound formed by the actions of Chirazyme and Amano PS in acetonitrile could be an unstable ester, which was isomerized by an intermolecular rearrangement to give *N*-methyl lauryoleathanolamide and other decomposed compounds upon silica gel column chromatography, evaporation, or in reactions conducted at higher temperatures.

The high reactivity of secondary amine functionality over the alcohol can be attributed to the higher nucleophilicity of the former. In polar solvents such as acetonitrile and dioxane, nucleophilicity is often enhanced and the hydroxyl group in these polar solvents may be involved in inter- or intramolecular H-bonds, thereby making it a poor nucleophile. Amino residues serve as a better nucleophiles than hydroxyl groups (22). However, an unstable by-product, presumably an ester that was rearranged to an amide, was formed in this study. Similar results of yielding a mixture of amide and mono-ester have been reported with the bifunctional substrate *N*-methyl-glucamine (with amine- and hydroxyl groups) and fatty acid methyl esters (21). The choices of the solvents forming secondary amides seem to be quite limited as we have seen in this study.

In conclusion, an efficient method has been developed for the Chirazyme-driven synthesis of the stable amide-bond surfactant *N*-methyl lauroylethanolamide from hydroxylated secondary amine and methyl laurate. This is one of the few examples of enzymatic secondary amide synthesis.

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