Enzymatic Synthesis of *N*-Acyl-L-Amino Acids in a Glycerol–Water System Using Acylase I from Pig Kidney

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ABSTRACT: *N*-Medium- and long-chain acyl-L-amino acids were enzymatically synthesized from the corresponding L-amino acids and fatty acids using a reverse hydrolysis. Enzymes that are suitable for the synthetic reaction of *N*-acyl-L-amino acids were screened on the basis of hydrolytic activity toward *N*-lauroyl-L-glutamic acid as an indicator. Acylase I from pig kidney (EC 3.5.1.14) showed the highest *N*-acyl-L-amino acid hydrolytic activity among 57 commercially available enzymes tested. Acylase I effectively catalyzed the synthesis of *N*-lauroyl-L-amino acids except for *N*-lauroyl-L-proline and *N*-lauroyl-L-tyrosine in a glycerol–water system. Under the optimized reaction conditions, *N*-lauroyl-L-arginine and *N*-lauroyl-L-glutamic acid were obtained in conversions of 82 and 44%, respectively. The equilibrium constants calculated from the conversion obtained were 5.6, 15.4, 18.0, and 39.4 for the syntheses of *N*-lauroyl-L-glutamic acid, N_{α}-lauroyl-L-lysine, *N*-lauroyl-L-glutamine, and *N*-lauroyl-L-methionine, respectively. *N*-Acyl-L-arginines with myristic acid and palmitic acid as the fatty acid were also synthesized using acylase I.

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KEY WORDS: *N*-acyl-L-amino acid, acylase I, enzymatic synthesis, pig kidney.

Surface-active agents are amphipathic molecules with hydrophilic and hydrophobic moieties and are used as surfactants. Surfactants constitute an important class of industrial chemicals that are widely used in the pharmaceutical, cosmetic, and food industries.

At present, most surfactants are chemically synthesized from petroleum as the raw material. However, developments over the past decade in enzyme technology in nonconventional media have suggested new possibilities for the synthesis of surfactants from natural sources (1). Many efforts have been devoted to enzyme- or microbial-catalyzed syntheses of biological surfactants such as monoglycerides, sugar fatty acid esters, lipoamino acids, phospholipids, and alkyl glycosides (1–3). Biological surfactants possess several advantages over the chemical surfactants, such as lower toxicity, higher biodegradability, and better environmental compatibility (2,3).

N-Acyl-L-amino acids, so-called lipoamino acids, are surfactants consisting of naturally occurring compounds: amino acids and fatty acids. *N*-Acyl-L-amino acids are known to exhibit excellent emulsifying properties and to possess strong antimicrobial activity (1). Such properties make *N*-acyl-L-amino acids attractive as food additives.

N-Acyl-L-amino acids are currently prepared by organic synthesis, mainly using amino acids and fatty acid chlorides with (4) or without using an organic solvent (5). However, the chemical synthetic method has the disadvantage that by-products, chloride salts, remain in the reaction mixture, which makes it necessary to remove the salt in order to retain a good detergency of the compounds. Thus, it is desired to develop a synthetic method using an enzyme.

A number of studies have been reported on the enzymatic synthesis of *N*-short-chain acyl-L-amino acids particularly using aminoacylase (6–9). It has been reported, for instance, that *N*-acetyl-L-methionine is synthesized effectively when the reaction mixture contains 3.2% water in ethyl acetate (8). On the other hand, *N*-medium- and long-chain acyl amino acids have been synthesized with immobilized lipases (10–13) using derivatives of fatty acids and amino acid as the substrates. However, in the synthetic methods adopted in these studies, the enzymatic synthesis is combined with a chemical synthesis; thus, *N*-acyl-L-amino acids were not obtained in one step.

In this paper, we synthesized various *N*-medium- and long-chain acyl-L-amino acids, using an enzyme, from the corresponding L-amino acids and fatty acids as the substrates. First, we screened the enzyme and an organic solvent suitable for the synthesis of *N*-medium- and long-chain acyl amino acids. The following variables were then investigated in order to find the optimal reaction conditions: pH, water content, and substrate concentrations.

MATERIALS AND METHODS

Materials. Aminoacylase (acylase I from pig kidney, 3500–5000 units/mg protein) was obtained from Wako Pure Chemical Industries (Osaka, Japan). For the other enzymes used in this study, their origins and suppliers are summarized in Table 1. Lipase A, Lipase AY, Lipase F-AP15, Lipase M, Lipase PS, Lipase R, Lipase R G, aminoacylase from *Aspergillus oryzae*, and Newlase F were kind gifts from Amano Pharmaceutical Co. (Aichi, Japan), Lipozyme IM and Novozyme 435 from Novo Nordisk Bioindustry, Ltd. (Chiba, Japan), and thermostable lipase from *Pseudomonas* sp. KWI-56 from Kurita Water Industries, Ltd. (Tokyo, Japan). *N*-Lauroyl-L-glutamic acid sodium salt, *N*-myristoyl-L-glutamic acid potassium salt, and *N*-stearoyl-L-glutamic acid sodium salt were kindly supplied by Ajinomoto

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TABLE 1
Hydrolysis of *N*-Lauroyl-L-glutamic Acid Hydrolyzed by Various Hydrolases

Enzyme	Origin	Supplier	Hydrolysis (%)
Lipase	*Aspergillus niger*	Fluka	—
	A. oryzae	Fluka	—
	Candida antarctica	Fluka	<5
	C. cylindracea	Fluka	—
	C. lipolytica	Fluka	—
	C. utilis	Fluka	<1
	C. rugosa	Sigma[a]	—
	Hog pancreas	Fluka	<1
	Human pancreas	Sigma[a]	—
	Mucor miehei	Fluka	<5
	M. javanicus	Fluka	<1
	Penicillium roqueforti	Fluka	<1
	Porcine pancreas	Sigma[a]	—
	Pseudomonas cepacia	Fluka	—
	P. fluorescens	Fluka	<1
	Rhizopus arrhizus	Fluka	<5
	R. niveus	Fluka	<1
	R. delemar	Fluka	<1
	Rhizomucor miehei	Fluka	<1
	Thermus aquaticus	Fluka	—
	T. flavus	Fluka	<1
	T. thermophilus	Fluka	—
	Type XII *Chromobacterium viscosum*	Sigma[a]	—
	Wheat germ	Fluka	—
Lipase A	*A. niger*	Amano[b]	<5
Lipase AY	*C. rugosa*	Amano[b]	—
Lipase F-AP15		Amano[b]	—
Lipase M	*M. javanicus*	Amano[b]	—
Lipase PS	*P. cepacia*	Amano[b]	<1
Lipase R	*Penicillium roqueforti*	Amano[b]	—
Lipase R G		Amano[b]	—
Lipozyme IM	*M. miehei*	Novo[c]	<5
Novozyme 435	*C. antarctica*	Novo[c]	<1
Thermostable lipase	*Pseudomonas* sp. KWI-56	Kurita[d]	—
Lipoprotein lipase	*Chromobacterium viscosum*	Fluka	<1
	Pseudomonas sp.	Fluka	—
	Pseudomonas sp. type B	Fluka	—
Acylase I[f]	Hog kidney	Fluka	~47
Acylase I[g]	Pig kidney	Wako	~77
Acylase I[h]	Porcine kidney	Sigma[a]	~62
Acylase I	*A. melleus*	Fluka	<5
Acylase I (immobilized)	*A. melleus*	Fluka	—
Aminoacylase	*A. oryzae*	Amano[b]	<5
Carboxypeptidase G	*Pseudomonas* sp.	Sigma[a]	~71
Carboxypeptidase P	*P. janthinellum*	Peptide Inst.[e]	<1
Newlase F	*Rhizopus niveus*	Amano[b]	—
Subtilisin	*Bacillus licheniformis*	Fluka	—
	Type XXIV Bacterial	Sigma[a]	—
	Type XXVII Bacterial	Sigma[a]	—
Protease	*Bacillus licheniformis*	Fluka	—
	Bacterial	Fluka	—
	Bacterial	Fluka	—
Esterase	*Bacillus* sp.	Fluka	<5
	Saccharomyces cerevisiae	Fluka	—
	Bacillus thermoglucosidasius	Fluka	—
	Thermoanaerobium brodcii	Fluka	—
	Horse liver	Fluka	—

[a]Sigma Chemical Co. (St. Louis, MO).
[b]Amano Pharmaceutical Co. (Aichi, Japan).
[c]Novo Nordisk Bioindustry, Ltd. (Chiba, Japan).
[d]Kurita Water Industries, Ltd. (Tokyo, Japan).
[e]Peptide Institute, Inc. (Osaka, Japan).
[f]15 U/mg, [g]71 U/mg, [h]42 U/mg. Here, 1 U corresponds to the amount of enzyme that produces 1 μmol of L-methionine per minute at pH 7.0 and 25°C.

Co., Inc. (Tokyo, Japan). *N*-Lauroyl-L-alanine, N_α-lauroyl-L-arginine, *N*-lauroyl-L-aspartic acid, N_α-lauroyl-L-glutamine, and N_α-lauroyl-L-lysine were purchased from Fluka Chemie AG (Neu Ulm, Germany). *N*-Lauroyl-L-glycine, *N*-lauroyl-L-serine, *N*-lauroyl-L-methionine, *N*-lauroyl-L-phenylalanine, and *N*-lauroyl-L-tryptophan were synthesized by the method of Lapidot *et al.* (14). Fatty acids and L-amino acids were obtained from Wako Pure Chemical Industries. All other chemicals were purchased from Wako Pure Chemical Industries.

Measurement of hydrolytic activity for N-*lauroyl-L-glutamic acid.* The reaction mixture contained 10 mM *N*-lauroyl-L-glutamic acid sodium salt, 50 mM Tris-HCl (pH 8.0), and 0.2% (wt/vol) enzyme powder in a final volume of 1.0 mL. The reaction mixture was incubated at 37°C for 3 h with magnetic stirring. Of 6 N HCl 100 μL was then added to the mixture to stop the reaction, followed by centrifugation. The amount of liberated glutamic acid in the supernatant was determined colorimetrically by the modified ninhydrin method (15), and the amount of *N*-lauroyl-L-glutamic acid hydrolyzed was then determined.

High-performance liquid chromatographic (HPLC) analysis of reaction products. The amount of product synthesized by the enzymatic reaction was measured using reversed-phase HPLC (RP-HPLC; PU-980, JASCO, Tokyo, Japan) with an ODS column (4.6 mm i.d. × 150 mm; R-ODS-5-ST, YMC Co., Kyoto, Japan), detected with a UV-Visible detector (UV-970, JASCO) at 200 nm. Elution was carried out using 40–65% (vol/vol) acetonitrile solution adjusted to pH 2.5 with phosphoric acid at a flow rate of 0.8 mL/min.

Selection of organic solvents for enzymatic synthesis. The effect of the addition of organic solvent on the conversion was examined using the standard reaction mixture containing 83 mM sodium L-glutamate, 5.6 mM lauric acid, 0.33% (wt/vol) acylase I, and 5 mL of cosolvent dissolved in 100 mM phosphate buffer, adjusted to pH 7.0 with 3 N NaOH, in a total volume of 6.0 mL. The final content of water as the phosphate buffer was 17% (vol/vol). The reaction mixture was preincubated at 37°C, followed by adding acylase I to start the reaction, and then incubated at 37°C for 24 h with magnetic stirring. The amount of the reaction product, *N*-lauroyl-L-glutamic acid, was determined by RP-HPLC, and the conversion was calculated on the basis of the initial molar concentration of lauric acid.

Enzymatic synthesis of N-*acyl-L-amino acids in a glycerol–water system.* Various amino acids and medium- and long-chain fatty acids were used as the substrates. The reaction mixture contained 0.083–1.0 M L-amino acid, 0.83–83 mM fatty acid, 0.33% (wt/vol) acylase I, and 3.0–5.75 mL of glycerol in 100 mM buffer solution, adjusted to pH 4.6–9.2, in a total volume of 6.0 mL. The final water content was 4.2–50% (vol/vol). The synthetic reaction was started at 37°C as described above, and the amount of product was determined by RP-HPLC. The reaction usually continued for 24 h with magnetic stirring. The conversion was calculated on the basis of the initial molar concentration of fatty acids.

Evaluation of equilibrium constants. The equilibrium constants of the synthetic reaction for *N*-lauroyl-L-amino acids were calculated from Equation 1.

$$K = \frac{[N\text{-lauroyl-L-amino acid}]_{eq}[H_2O]}{[\text{L-amino acid}]_{eq}\ [\text{lauric acid}]_{eq}} \quad [1]$$

The term $[\ ]_{eq}$ denotes the equilibrium concentration of the components. The *N*-lauroyl-L-amino acid concentration was determined by RP-HPLC as described above. The concentrations of L-amino acid and lauric acid were calculated by subtracting the product concentration from the initial values. The change in the water content during the reaction was no more than 0.05% and was neglected in the calculation of the equilibrium constant.

In the case of *N*-lauroyl-L-arginine, the product tended to precipitate in the course of the synthetic reaction. The solubility (saturated molar concentration) of *N*-lauroyl-L-arginine was in the order of 0.2 mM. Thus, $[N\text{-lauroyl-L-amino acid}]_{eq}$ in Equation 1 corresponds to the solubility of *N*-lauroyl-L-arginine and could be assumed to be a constant. Therefore, Equation 1 gives Equation 2 for the case of *N*-lauroyl-L-arginine as

$$K_{SP} = \frac{[H_2O]}{[\text{L-amino acid}]_{eq}\ [\text{lauric acid}]_{eq}} \quad [2]$$

where $K_{SP} = K/[N\text{-lauroyl-L-arginine}]_{sat}$ and $[N\text{-lauroyl-L-arginine}]_{sat}$ indicates the saturated molar concentration.

Preparation of N-*lauroyl-L-glutamic acid in a gram scale.* A half mol (93.6 g) of sodium L-glutamate and 0.0042 mol (0.84 g) of lauric acid were suspended in 62.5 mL of 100 mM phosphate buffer, pH 7.5. Then 437.5 mL of glycerol was added, and finally the pH of the solution was readjusted to 7.5. The mixture was preincubated at 37°C, and then 0.5 g of acylase I was added at 0.1% (wt/vol) to start the reaction. The reaction was allowed to continue at 37°C for 66 h with gentle shaking. After the reaction, the reaction mixture was heated at 90°C for 15 min to inactivate the enzyme. The reaction mixture was diluted two times with distilled water and its pH was adjusted to 5.5 with 6 N HCl. The reaction solution was mixed with an equal volume of *n*-hexane with shaking to extract lauric acid from the reaction solution; the extraction was repeated twice. The pH of the aqueous layer was then adjusted to 1.0 with 6 N HCl followed by extraction of the product into 700 mL of ethyl acetate, and the extraction was repeated. The ethyl acetate phase was evaporated to dryness using a rotary evaporator and the residue was dissolved in 50 mL of 3 N KOH. This alkaline solution was acidified to pH 1.0 with 15% (vol/vol) sulfuric acid (5) and cooled to around 4°C to precipitate the product. The precipitated product was separated by centrifugation and washed with distilled water several times. The dried powder was subjected to analyses by ^{1}H nuclear magnetic resonance (NMR) and RP-HPLC to analyze the structure and purity.

RESULTS AND DISCUSSION

Screening of hydrolases. The synthesis reaction of an *N*-acyl-L-amino acid from an amino acid and a fatty acid is the reverse of hydrolysis. Therefore, it could be considered that the enzyme catalyzing the hydrolysis would catalyze the synthesis of *N*-acyl-L-amino acid. Thus, hydrolases with high hydrolytic activity toward *N*-lauroyl-L-glutamic acid were chosen for use in the synthetic reaction. We used commercially available enzymes such as acylase I, lipase, and protease (Table 1). The reason why we tested the enzymes shown in Table 1 is as follows: Acylase I is known to catalyze the reverse reaction as well as the hydrolysis of *N*-short-chain acyl L-amino acids (6–9) and lipases as well as proteases catalyze the synthesis of the amide bond in an organic solvent (10–13,16,17).

As shown in Table 1, acylase I from pig kidney showed the highest hydrolytic activity toward *N*-lauroyl-L-glutamic acid among 57 enzymes examined. The degree of hydrolysis of *N*-lauroyl-L-glutamic acid was 77% with acylase I. On the other hand, protease, esterase, and lipase showed scarcely any hydrolytic activity. In this study, we used acylase I for the synthesis of *N*-acyl-L-amino acids in the subsequent experiments.

Examination of organic solvents for enzymatic synthesis. The fundamental problem in applying the hydrolases to the synthetic reaction is how to shift the equilibrium toward the condensation product. Usually, addition of organic solvents, water-miscible or immiscible, in the reaction media is effective not only to shift the equilibrium but also to dissolve more reactants in the medium. First, we tested water-immiscible organic solvents such as ethyl acetate, *n*-butanol, and *n*-hexane. However, the product was nearly undetectable in both phases probably because the product was not partitioned into the organic solvent phase. On the other hand, addition of some water-miscible organic solvents [83% (vol/vol)] was effective in stimulating the enzymatic synthesis of *N*-acyl-L-amino acids. Table 2 shows the effect of the addition of water-miscible organic solvents on the synthesis of *N*-lauroyl-L-glutamic acid during 24-h incubation at 37°C. Synthesis was increased by addition of the organic solvent except for methanol, ethanol, and acetone. When methanol, ethanol, and acetone were added, the product was not detected, probably because of inactivation of the enzyme. The conversion was greatly increased (by 30-fold) in a glycerol–water system compared to that in the buffer system. Homandberg *et al.* (18) showed that the addition of an organic cosolvent such as glycerol could increase the equilibrium for peptide bond formation from the acid and amine components of the amino acid derivatives owing to the decreased dielectric constant. Thus, a similar mechanism could be considered to be the reason for the remarkable increase in the conversion of *N*-lauroyl-L-glutamic acid by the addition of glycerol (Table 2), although the details are not known at present. Subsequent experiments were carried out accordingly in the glycerol–water system.

Optimization of reaction conditions for synthesis of N-*lauroyl-L-amino acids.* The enzymatic synthesis of *N*-lauroyl-L-amino acids was performed in a glycerol–water system using acylase I under various conditions. It was found that acylase I catalyzed the synthesis of *N*-lauroyl-L-amino acids from lauric acid and various amino acids except for L-proline and L-tyrosine (data not shown). Probably, *N*-lauroyl-L-proline was not synthesized because of the cyclic structure of proline.

The effect of pH on the reaction was examined in a glycerol–water system with a water content of 17% (vol/vol) using

TABLE 2
Synthesis of *N*-Lauroyl-L-glutamic Acid in Water-Miscible Organic Solvents

Cosolvents	Synthesis[a] (%)
Control[b]	0.19
Acetone	—[c]
Acetonitrile	0.46
N,N-Dimethylformamide	0.31
Methanol	—[c]
Ethanol	—[c]
1-Propanol	0.54
1,4-Butanediol	1.64
Ethylene glycol	1.33
Diethylene glycol	0.84
Triethylene glycol	1.56
Polyethylene glycol 300	2.60
Glycerol	6.43

[a]The reaction time was 24 h. The synthesis was calculated on the basis of the initial molar concentration of lauric acid.
[b]The reaction was carried out in 100 mM phosphate buffer (pH 7.0) without adding a cosolvent.
[c]Not detected.

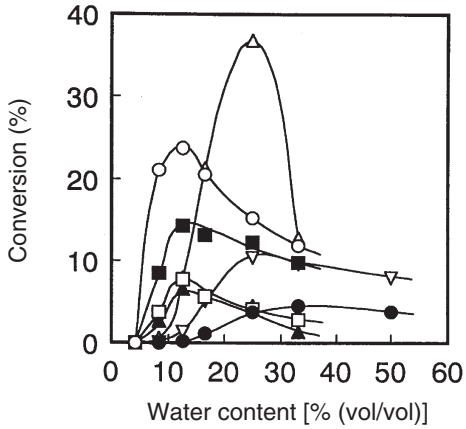

FIG. 2. Effect of water content in the reaction mixture on the conversion. The reaction mixture, containing 4.2–50% (vol/vol) 100 mM buffer (pH 7.5), 83 mM amino acid, 5.6 mM lauric acid, glycerol, and 0.33% (wt/vol) acylase I in a total volume of 6.0 mL, was incubated at 37°C for 24 h under magnetic stirring. (○), L-Methionine; (●), L-lysine; (□), L-glutamic acid; (■), L-glutamine; (△), L-arginine; (▲), L-alanine; (▽), L-histidine.

83 mM sodium L-glutamate, L-methionine, and L-lysine hydrochloride and 5.6 mM lauric acid as the substrates. The synthetic reaction reached nearly equilibrium within 24 h (data not shown). Figure 1 shows the relationship between pH and the conversions after 24 h for the syntheses of *N*-lauroyl-L-glutamic acid, *N*-lauroyl-L-methionine, and N_α-lauroyl-L-lysine. N_ε-Lauroyl-L-lysine was not produced since acylase I specifically recognizes the alpha amino group of L-lysine (19). As shown in Figure 1, the optimal pH for the syntheses of these *N*-acyl-L-amino acids was around 7.0–7.5.

Figure 2 shows the effect of water content in the reaction mixture on the conversion after 24 h at pH 7.5 using 83 mM amino acids and 5.6 mM lauric acid. The synthetic reaction nearly reached equilibrium within 24 h. There was an optimal water content for the synthesis of *N*-lauroyl-L-amino acids in the glycerol–water system, and it differed with the kind of amino acid. The optimal water contents for the syntheses of *N*-lauroyl-L-methionine, *N*-lauroyl-L-glutamine, *N*-lauroyl-L-glutamic acid, and *N*-lauroyl-L-alanine were around 12.5%. However, in the syntheses of *N*-lauroyl-L-arginine, *N*-lauroyl-L-histidine, and N_α-lauroyl-L-lysine, the amino acid moieties of which were basic, the optimal water content was higher than 12.5%, although the reason is not known. Usually, a low water content in the reaction mixture is more favorable for shifting the equilibrium toward the synthetic reaction. It has been reported, for instance, that *N*-acetyl-L-methionine is synthesized effectively when the reaction mixture contains 3.2% water in ethyl acetate (8). The conversions for *N*-lauroyl-L-amino acids, however, were sharply decreased in the range of water content lower than around 10%, as shown in Figure 2. Hydrophilic solvents such as glycerol used in this study tend to remove water molecules existing around the surface of the enzyme molecule, which lowers the enzyme activity.

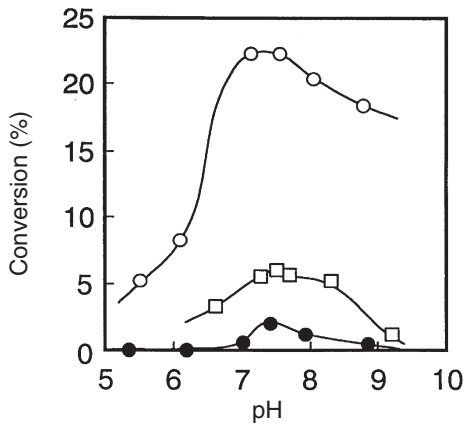

FIG. 1. Effect of pH on the synthetic reaction. pH dependences of the conversion of *N*-lauroyl-L-methionine (○), *N*-lauroyl-L-lysine (●), and *N*-lauroyl-L-glutamic acid (□). The reaction mixture, containing 17% (vol/vol) 100 mM buffer (pH 4.63–9.20), 83 mM amino acid, 5.6 mM lauric acid, glycerol, and 0.33% (wt/vol) acylase I in a total volume of 6.0 mL, was incubated at 37°C for 24 h under magnetic stirring.

The effect of the substrate concentration on the conversion was examined using 0.83–83 mM lauric acid at 83 mM sodium L-glutamate. The conversion was nearly constant in the lauric acid concentration range of 2 to 10 mM and was decreased at 10 mM and higher (data not shown). It was suggested that the solubility of lauric acid reached saturation at around 10 mM. Figures 3A and 3B illustrate the effect of amino acid concentration on the equilibrium conversion to *N*-lauroyl-L-amino acids. In every case, the reaction was allowed to continue until the equilibrium was confirmed. In the case of *N*-lauroyl-L-arginine synthesis, the equilibrium conversion was over 80%, probably because the solubility of the product was very low (around 2×10^{-4} M). The equilibrium conversion of *N*-lauroyl-L-glutamic acid increased with the increasing concentration of amino acid and reached around 40% at 1.0 M. On the other hand, those of *N*-lauroyl-L-glutamine, *N*-lauroyl-L-methionine, and N_α-lauroyl-L-lysine increased at the low concentration range of amino acids and did not change with a further increase in the concentration, probably because of low sol-

ubility of the amino acids. The change in the equilibrium conversion with amino acids is discussed later.

Equilibrium constants. The equilibrium constants for the synthesis of *N*-lauroyl-L-amino acids were determined from the plots in Figures 3A and 3B to fit the experimental results to those calculated from Equations 1 and 2. The equilibrium constants determined using Equation 1 were 5.6, 15.4, 18.0, and 39.4 for the syntheses of *N*-lauroyl-L-glutamic acid, N_α-lauroyl-L-lysine, *N*-lauroyl-L-glutamine, and *N*-lauroyl-L-methionine, respectively. In the case of N_α-lauroyl-L-lysine synthesis, the powder enzyme was added three times (20 mg each) during a 200-h reaction because of its low reaction rate. The conversions of *N*-lauroyl-L-glutamic acid and N_α-lauroyl-L-lysine calculated using the equilibrium constants were in good agreement with the experimental results over the whole concentration range as shown in Figure 3A and 3B. However, the experimental conversions for *N*-lauroyl-L-methionine and *N*-lauroyl-L-glutamine deviated from the calculated values when the amino acid concentration exceeded 0.1 M. The reason for this deviation could be explained by the fact that the solubilities of L-glutamine and L-methionine were saturated at around 0.1 M.

The synthetic rate of *N*-lauroyl-L-arginine was low, and the enzyme powder was added twice in a way similar to that for N_α-lauroyl-L-lysine. The K_{SP} value shown in Equation 2 for *N*-lauroyl-L-arginine was determined from the plots in Figure 3A to be 8.2×10^4 [M^{-1}]. As shown in Figure 3A, the theoretical conversions of *N*-lauroyl-L-arginine were coincident with the experimental results at the amino acid concentrations lower than around 0.1 M. At the amino acid concentrations higher than 0.25 M, there were deviations between the calculated and experimental results although the reason is not known.

The conversion of *N*-lauroyl-L-arginine was quite high because the product was insoluble. The equilibrium constants of *N*-lauroyl-L-methionine and *N*-lauroyl-L-glutamine were larger than that of *N*-lauroyl-L-glutamic acid. However, the conversion was not high, because the solubilities of L-methionine and L-glutamine in a glycerol–water system were lower than that of L-glutamic acid. Therefore, it was suggested that high solubility was required to obtain *N*-lauroyl-L-methionine and *N*-lauroyl-L-glutamine in high conversion.

Substrate specificity for the synthesis of N-*acyl-L-amino acids.* Table 3 summarizes the synthesis of *N*-lauroyl-L-amino acids with various amino acids as the substrate under nearly optimized conditions. The reaction nearly reached equilibrium. *N*-Lauroyl-L-aspartic acid was scarcely synthesized despite having a structure similar to that of *N*-lauroyl-L-glutamic acid. This result is in agreement with the fact that *N*-acyl-L-aspartic acid is not hydrolyzed by acylase I (19). *N*-Lauroyl-L-phenylalanine was not obtained in a large amount in the glycerol–water system, although it was produced in a conversion of 22% in the buffer solution. *N*-Lauroyl-L-valine and *N*-lauroyl-L-leucine showed a tendency similar to that of *N*-lauroyl-L-phenylalanine.

The synthesis of *N*-acyl-L-arginine and *N*-acyl-L-glutamic acids with various fatty acids as the substrate is given in Figure 4. The synthetic reactions for *N*-lauroyl-L-arginine and *N*-lauroyl-L-glutamic acid were conducted for 144 and 24 h, respectively. The conversions of *N*-acyl-L-glutamic acid were around 40–50% using fatty acids with a chain length between 8 and 12, whereas the conversions were considerably decreased by increasing the chain length over 13. On the other hand, *N*-acyl-L-arginine showed quite a different tendency. The conversion of *N*-acyl-L-arginine was extremely high for fatty acids with chain lengths of 12 and 14, while the conversions using the other fatty acids were low compared with those using lauric acid and myristic acid. Although *N*-palmitoyl-L-arginine was synthesized, *N*-palmitoyl-L-glutamic acid was not. These findings might suggest that acylase I shows a different specificity toward the fatty acid depending on the kind of amino acid. Thus, we showed for the first time that *N*-medium- and long-chain acyl-L-amino acids could be synthesized using acylase I from pig kidney using natural resources, amino acids and fatty acids.

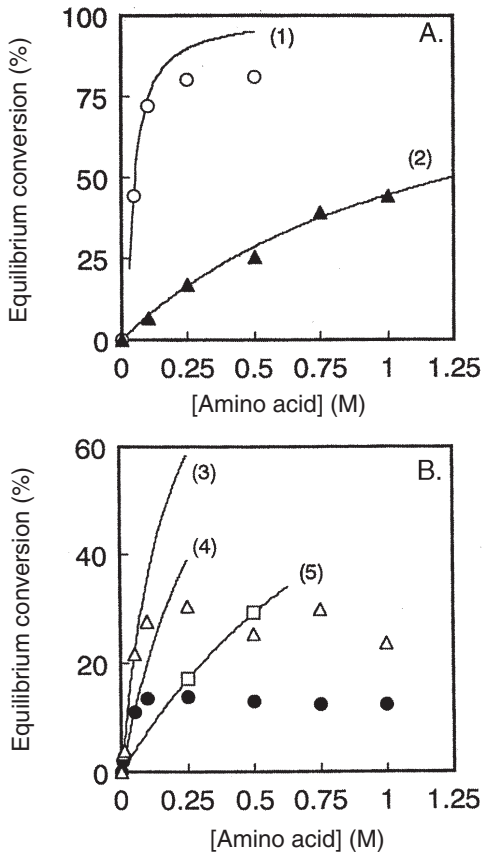

FIG. 3. Effect of amino acid concentration on the conversion of *N*-lauroyl-L-amino acids. The reaction mixture, containing 12.5% (vol/vol) 100 mM buffer (pH 7.5), 0.005–1.0 M amino acid, 6.2 or 8.3 mM lauric acid, glycerol, and 0.33% (wt/vol) acylase I in a total volume of 6.0 mL, was incubated at 37°C under magnetic stirring for syntheses of *N*-lauroyl-L-glutamine, -L-glutamic acid, -L-methionine. For syntheses of *N*-lauroyl-L-arginine and -L-lysine, the water content was 25 and 33% (vol/vol) with an acylase I concentration of 1 and 1.33% (wt/vol), respectively. The experimental conversions: (A), (○), L-Arginine; (▲), L-glutamic acid, (B), (△), L-methionine; (□), L-lysine; (●), L-glutamine. The solid lines (1)–(5) represent the calculated equilibrium conversions of *N*-lauroyl-L-amino acids: (A), (1), L-Arginine; (2), L-glutamic acid, (B), (3), L-methionine; (4), L-glutamine; (5), L-ly-

Preparative enzymatic synthesis of N-*lauroyl-L-glutamic acid. N*-Lauroyl-L-glutamic acid was prepared in a gram scale under the conditions described in the Materials and Methods

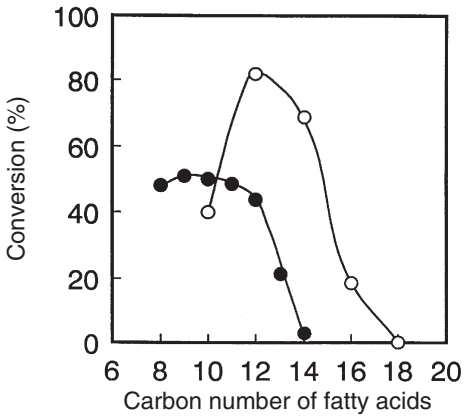

FIG. 4. Synthesis of *N*-acyl-L-arginines and *N*-acyl-L-glutamic acids with various fatty acids as the substrate. The reaction mixture for *N*-acyl-L-arginines, containing 25% (vol/vol) 100 mM buffer (pH 7.5), 0.5 M L-arginine hydrochloride, 6.2 mM fatty acid, glycerol, and 0.33% (wt/vol) acylase I in a total volume of 6.0 mL, was incubated at 37°C for 144 h under magnetic stirring. In the case of *N*-acyl-L-glutamic acids, the reaction mixture, containing 12.5% (vol/vol) 100 mM buffer (pH 7.5), 1.0 M sodium L-glutamate, 8.3 mM fatty acid, glycerol, and 0.33% (wt/vol) acylase I in a total volume of 6.0 mL, was incubated for 24 h. (○), L-Arginine; (●), L-glutamic acid.

section. After the reaction, the conversion for *N*-lauroyl-L-glutamic acid was found to be 27.4% by an analysis using RP-HPLC on the basis of the initial molar concentration of lauric acid, which was somewhat lower than that obtained in a small scale (*ca.* 44%). After extraction and precipitation, the weight of the dried powder product was 0.31 g with the purity higher than 97% analyzed by RP-HPLC. The yield of *N*-lauroyl-L-glutamic acid was calculated to be 22.7% on the basis of molar scale.

The product was also analyzed by ^{1}H NMR. The analysis indicated that lauric and glutamic acids were condensed at a molar ratio of 1:1. ^{1}H NMR (300 MHz, CD_3OD, TMS, 298K): δ 0.89 (3 H, *t*, *J* = 7.0 Hz), 1.29 (16 H, *m*), 1.61 (2 H, *t*, *J* = 7.0 Hz), 1.93 (1 H, *m*), 2.16 (1 H, *m*), 2.24 (2 H, *t*, *J* = 7.2 Hz), 2.39 (2 H, *t*, *J* = 7.7 Hz), 4.42 (1 H, *dd*, J_1 = 5.0 Hz, J_2 = 9.1 Hz).

TABLE 3
Conversion of *N*-Lauroyl-L-amino Acids with Various Amino Acids as the Substrate[a]

Amino acid moiety	Conversion (%)	Amino acid moiety	Conversion (%)
L-Aspartic acid	0.9	L-Asparagine	25.1
L-Glutamic acid	44.4	L-Glutamine	13.8
L-Lysine	29.3	L-Alanine	20.7
L-Arginine	81.8	L-Valine	22.4
L-Histidine	11.0	L-Leucine	12.1
L-Glycine	23.8	L-Isoleucine	4.6
L-Serine	35.1	L-Methionine	30.5
L-Threonine	19.8	L-Phenylalanine	3.3
L-Cysteine	8.8		

[a]The lauric acid concentration in the case of the amino acid moieties of L-lysine, L-arginine, L-histidine, and L-methionine and that in the other amino acid moieties were 6.2 and 8.3 mM, respectively. The concentrations of L-amino acids were 0.5 M, except for L-glutamic acid (1.0 M). The reaction was carried out with a water content of 12.5 % (vol/vol) in glycerol for all amino acids except L-arginine (25%), L-histidine (25%), and L-lysine (33 %). The conversion was calculated on the basis of the initial molar concentration of the fatty acid.

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REFERENCES

1. Sarney, D.B., and E.N. Vulfson, Application of Enzymes to the Synthesis of Surfactants, *Trends Biotechnol. 13*:164–172 (1995).
2. Desai, J.D., and I.M. Banat, Microbial Production of Surfactants and Their Commercial Potential, *Microb. Mol. Biol. Rev. 61*:47–64 (1997).
3. Fiechter, A., Biosurfactants: Moving Toward Industrial Application, *Trends Biotechnol. 10*:208–217 (1992).
4. Desai, A., A. George, and P. Bahadur, Surfactant Properties of Disodium Salts of *N*-Acyl-aspartic and Glutamic Acids, *Tenside Surf. Det. 36*:96–101 (1999).
5. Hattori, T., and K. Hirai, Process for Producing *N*-Long-Chain Acyl Acidic Amino Acids or Salts Thereof, Eur. Pat. Appl. EP 827,950 (1998).
6. Ferjancic-Biagini, A., T. Giardina, M. Reynier, and A. Puigserver, Hog Kidney and Intestine Aminoacylase-Catalyzed Acylation of L-Methionine in Aqueous Media, *Biocatal. Biotransform. 15*: 313–323 (1997).
7. Kosáry, J., C.S. Sisak, B. Szajani, and L. Boross, Acylation of Amino Acids by Aminoacylase in Non-conventional Media, *Biocatalysis 11*:329–337 (1994).
8. Yokoigawa, K., E. Sato, N. Esaki, and K. Soda, Enantioselective Synthesis of *N*-Acetyl-L-methionine with Aminoacylase in Organic Solvent, *Appl. Microbiol. Biotechnol. 42*:287–289 (1994).
9. Kimura, Y., Y. Kobayashi, S. Adachi, and R. Matsuno, Aminoacylase-Catalyzed Synthesis of *N*-Acyl Amino Acid from Fatty Acid or Its Ethyl Ester and Amino Acid, in *Biochemical Engineering 2001. Proc. Asia-Pac. Biochem. Eng. Conf.*, edited by S. Furusaki, I. Endo, and R. Matsuno, Springer, Tokyo, 1992, pp. 109–111.
10. Vonderhagen, A., H.-C. Raths, and E. Eilers, Enzymatic Catalyzed N-Acylation of Amino Acids, Protein Hydrolysates and/or Their Derivatives, Ger. Offen. DE 19,749,555 (1999).
11. Vonderhagen, A., H.-C. Raths, and E. Eilers, Enzymatic Catalyzed N-Acylation of Amino Acids, Protein Hydrolysates and/or Their Derivatives, Ger. Offen. DE 19,749,556 (1999).
12. Montet, D., F. Servat, M. Pina, J. Graille, P. Galzy, A. Arnaud, H. Ledon, and L. Marcou, Enzymatic Synthesis of *N*-ε-Acyllysines, *J. Am. Oil Chem. Soc. 67*:771–774 (1990).
13. Godtfredsen, S.E., and F. Bjoerkling, An Enzyme-Catalyzed Process for Preparing *N*-Acylamino Acids and *N*-Acylamino Acid Amides, PCT Int. Appl. WO 90 14,429 (1990).
14. Lapidot, Y., S. Rappoport, and Y. Wolman, Use of Esters of *N*-Hydroxysuccinimide in the Synthesis of *N*-Acylamino Acids, *J. Lipid Res. 8*:142–145 (1967).
15. Moore, S., and W.H. Stein, A Modified Ninhydrin Reagent for the Photometric Determination of Amino Acids and Related Compounds, *J. Biol. Chem. 211*:907–913 (1954).
16. Clapés, P., C. Morán, and M.R. Infante, Enzymatic Synthesis of Arginine-Based Cationic Surfactants, *Biotechnol. Bioeng. 63*: 333–343 (1999).
17. Gadossi, L., D. Bianchi, and A.M. Klibanov, Selective Acylation of Peptides Catalyzed by Lipases in Organic Solvents, *J. Am. Chem. Soc. 113*:6328–6329 (1991).
18. Homandberg, G.A., J.A. Mattis, and M.J. Laskowski, Synthesis of Peptide Bonds by Proteinases. Addition of Organic Co-solvents Shifts Peptide Bond Equilibria Toward Synthesis, *Biochemistry 17*:5220–5227 (1978).
19. Anders, M.W., and W. Dekant, Aminoacylase, *Adv. Pharmacol. 27*:431–448 (1994).

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