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A Novel Acylase from *Streptomyces mobaraensis* that Efficiently Catalyzes Hydrolysis/Synthesis of Capsaicins as Well as *N*-Acyl-L-amino Acids and *N*-Acyl-peptides

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A novel enzyme that catalyzes efficient hydrolysis of capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) was isolated from the culture broth of *Streptomyces mobaraensis*. The enzyme consisted of two dissimilar subunits with molecular masses of 61 and 19 kDa. The enzyme was activated and stabilized in the presence of Co^{2+}. It showed a pH optimum of about 8 and was stable at temperatures of up to 55 °C for 1 h at pH 7.8. The specific activity of the enzyme for the hydrolysis of capsaicin was 10^2-10^4 times higher than those for the enzymes reported to date. In an aqueous/*n*-hexane biphasic system, capsaicin analogues such as octanoyl, decanoyl, and lauroyl vanillylamides were synthesized from the corresponding fatty acids and vanillylamine at yields of 50% or greater. In addition, the enzyme catalyzed the deacylation of *N*-lauroyl-L-amino acids and *N*-lauroyl-L-dipeptides and the efficient synthesis of *N*α-lauroyl-L-lysine, *N*ε-lauroyl-L-lysine, and various *N*-lauroyl-peptides in aqueous solution in both the absence and the presence of glycerol.

KEYWORDS: Capsaicin; actinomycetes; *Streptomyces mobaraensis*; lauric acid; capsaicin derivative; *N*-acyl-L-amino acid; *N*-acyl-peptide

INTRODUCTION

Capsaicin, an amide comprised of vanillylamine (4-hydroxy-3-methoxybenzylamine) and 8-methyl-6-*trans*-nonenoic acid, is a pungent compound produced by chili peppers and related plants of the *Capsicum* family. Capsaicin has a variety of biological activities such as enhancement of energy metabolism, stimulation of gastric acid secretion, and the analgesic activity (*1*−*3*). It has also been reported that the extent of analgesia and pungency is altered for capsaicin derivatives that contain fatty acyl moieties of various chain lengths (*1*, *4*, *5*). Thus, several investigators have reported on the synthesis of capsaicin analogues and have evaluated their analgesic activities (*6*, *7*).

The aim of this study was to synthesize capsaicin analogues enzymatically, since an enzymatic method has various advantages over chemical methods such as permitting the reaction to be conducted under mild conditions and the repression of side reactions. One approach to this would be to synthesize capsaicin analogues from vanillylamine and fatty acids using the reverse reaction of hydrolysis, using a capsaicin-hydrolyzing enzyme in an organic solvent, similar to the procedures used to prepare peptide precursors using proteases (*8*, *9*). We report herein on the purification/characterization of a novel acylase from *Streptomyces mobaraensis* NBRC (IFO) 13819 that principally catalyzes the hydrolysis of capsaicin and synthesis of capsaicin derivatives.

MATERIALS AND METHODS

Materials. Capsaicin was obtained from Sigma-Aldrich Co. (St. Louis, MO). Beef extract, malt extract, and yeast extract were obtained from Difco Laboratories (Detroit, MI). Polypepton was purchased from Nihon Pharmaceutical Co., Ltd. (Tokyo, Japan). NZ amine (type A) and soluble starch were obtained from Wako Pure Chemical Industries (Osaka, Japan). CM Sephadex C-50 ion exchange gel and hydroxyapatite gel (fast flow type) were products of Amersham Pharmacia Biotech (Uppsala, Sweden) and Wako, respectively. *N*-Acetyl-L-amino acids were purchased from either Sigma-Aldrich Co. or Wako. *N*-Lauroyl-L-amino acids and *N*-lauroyl-L-dipeptides were synthesized by reacting the *N*-hydroxysuccinimide ester of lauric acid with amino acids or dipeptides [purchased from Wako, the Peptide Institute, Inc. (Osaka), and Kokusan Chemical Co. (Tokyo)] according to the method of Lapidot et al. (*10*). *N*α-Lauroyl-L-lysine and *N*ε-lauroyl-L-lysine were obtained from either Sigma-Aldrich Co. or the Ajinomoto Co., Inc. (Tokyo). Vanillylamine was purchased from Sigma-Aldrich Co. Octanoic acid, decanoic acid, lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and *n*-hexane were obtained from Wako. Diglycine and tripeptides were purchased from either Sigma-

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Table 1. Capsaicin-Hydrolyzing Activities for 21 Strains of *Streptomyces* sp.

	NBRC no.	activity (U/mL)
S. mobaraensis	13819	1.2 ± 0.1
S. ardus	13430	
S. blastmyceticus	12747	
S. cacaoi	13813	
S. caespitosus	13490	
S. cinnamoneus	12852	
S. exfoliatus	12319	
S. griseinus	12869	
S. lividoclavatus	13870	
S. lividus	13787	
S. luteoreticuli	13422	1.0
S. mobaraenis	13476	
S. olivaceus	12805	
S. roseoverticillatus	12817	
S. scabiei	13767	
S. sioyaensis	12820	
S. spheroides	12917	
S. toyocaensis	12824	
S. tuirus	15617	
S. venezuelae	13097	
S. violaceoruber	13385	

Aldrich Co. or the Peptide Institute. All other reagents were of analytical grade and purchased from either Wako or Nacalai Tesque, Inc. (Kyoto, Japan).

Buffers. The following buffers were used in this study. Buffer A: 50 mM Tris-HCl, pH 7.8; buffer B: 25 mM Tris-HCl, pH 7.5; buffer C: 40 mM potassium phosphate, pH 7.5; buffer D: 100 mM Tris-HCl containing 0.5 mM $CoCl_2$, pH 6.9; and buffer E: 100 mM Tris-HCl containing 0.5 mM $CoCl_2$, pH 7.5. Buffer A was used for the enzyme assay, and buffers B and C were used, respectively, as elution buffers in CM Sephadex C-50 and hydroxyapatite gel chromatographies. Buffers D and E were utilized in synthetic reactions of capsaicin analogues and *N*-lauroyl-L-amino acids/*N*-lauroyl-peptides, respectively.

Bacteria and the Cultivation of Cells. *S. mobaraensis* NBRC13819 (National Institute of Technology and Evaluation Biological Resource Center, Chiba, Japan) is typically used for the production of an acylase enzyme. In addition, we tested 20 *Streptomyces* strains listed in **Table 1**. Each strain was aseptically transferred to an agar plate (4 g of yeast extract, 10 g of malt extract, 4 g of glucose, and 20 g of agar in 1 L of water, adjusted to pH 7.3) and statically incubated for 7 days at 30 °C. A loopful of the agar culture was then incubated in a 300 mL shaking flask containing 30 mL of preculture medium [10 g of glucose, 10 g of dextrin, 5 g of NZ amine (type A), 5 g of yeast extract, and 1 g of $CaCO_3$ in 1 L of water, adjusted to pH 6.5] and incubated at 30 °C with reciprocal shaking at 120 strokes/min. A 1.8 mL aliquot of the preculture was added to 500 mL shaking flasks containing 50 mL of medium (40 g of beef extract, 40 g of soluble starch, 20 g of Polypepton, 2 g of K_2HPO_4, and 20 g of $MgSO_4$ in 1 L of water, adjusted to pH 7.0) for the main culture, which was the optimal medium found. The cells were grown at 30 °C with reciprocal shaking at 120 strokes/min for 8 days. After cultivation, the culture broth was recovered by centrifugation at 20000*g* for 30 min at 4 °C.

Enzyme Assay. The capsaicin hydrolytic activity was measured as follows. A 20 μL aliquot of the enzyme solution was added in 180 μL of capsaicin solution dissolved in buffer A at a final concentration of 130 μM and incubated for 20 min at 37 °C. The remaining capsaicin concentration in the reaction mixture was determined by high-performance liquid chromatography (HPLC) (Shimadzu, Corp., Kyoto) using a 5C18-AR−II column (4.6 mm i.d. × 150 mm, Nacalai Tesque, Inc.) with detection at 280 nm. Elution was carried out using 50% (v/v) acetonitrile solution containing 0.075% phosphoric acid as the mobile phase at a flow rate of 0.8 mL/min at room temperature. One unit of capsaicin-hydrolyzing activity was arbitrarily defined as the amount of the enzyme required to hydrolyze 1 μmol of capsaicin in 1 h at 37 °C at pH 7.8. Protein concentrations were determined using a BCA Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL) with bovine serum albumin (BSA) as a standard.

Purification of Capsaicin-Hydrolyzing Enzyme. All purification procedures were carried out at 4 °C. Ammonium sulfate was first added to the culture supernatant (800 mL) to 60% saturation to precipitate soluble proteins. The resulting precipitate was separated by centrifugation at 20000*g* for 30 min and dissolved in 100 mL of buffer B containing 50 mM NaCl and dialyzed against the same buffer. The dialyzed solution was placed on a CM Sephadex C-50 gel column (1.6 cm i.d. × 35 cm) equilibrated with the same buffer as was used for the dialysis. Elution was accomplished by a linear increase in the NaCl concentration in buffer B from 50 to 500 mM at a flow rate of 0.35 mL/min. Fractions showing capsaicin-hydrolyzing activity were collected, concentrated, and dialyzed against buffer C. The enzyme solution was applied to a hydroxyapatite gel column (1.6 cm i.d. × 15 cm) and eluted by linearly increasing the concentration of potassium phosphate buffer, pH 7.5, from 40 to 400 mM at a flow rate of 0.18 mL/min. Active fractions were collected, concentrated, and finally dialyzed against buffer A.

Sodium Dodecyl Sulfate−Polyaclylamide Gel (SDS−PAGE) and Native PAGE. SDS−PAGE was performed using a 12.5% gel (Bio-Rad Laboratories, Mini-Protean III Ready Gels J, Hercules, CA) by the method of Laemmli (*11*). The protein bands were stained with Coomassie Brilliant Blue R-250 (CBB R-250, Sigma-Aldrich Co.). Phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (Amersham Pharmacia Biotech, LMW Electrophoresis Calibration Kit) were used as standard proteins. Native PAGE was carried out using a 5−20% nondenatured gradient gel (Bio-Rad Laboratories, Mini-Protean III Ready Gels J) in which an acetic acid/β-alanine buffer, pH 4.5, was used as a reservoir buffer according to Reisfeld et al. (*12*) to estimate the molecular mass of the enzyme. As marker proteins for native PAGE, glyceraldehydes-3-phosphate dehydrogenase from rabbit muscle (145 kDa), alcohol dehydrogenase from equine liver (79.5 kDa), avidin from egg white (60 kDa), trypsin from bovine pancreas (23 kDa), and ribonuclease A from bovine pancreas (14 kDa) were used, all of which were obtained from Sigma-Aldrich Co. Protein bands were stained with CBB R-250. The molecular mass of the enzyme was determined from a calibration curve prepared from the mobility of the marker proteins vs the logarithms of their molecular masses.

Effects of Reagents and Metals on the Stability of Capsaicin-Hydrolyzing Enzyme. A solution of the enzyme in buffer A was preincubated at 37 °C for 15 min in the presence of a final 1 mM concentration of reagents and metals [*p*-chloromercuribenzoic acid (PCMB), iodoacetamide, dithiothreitol (DTT), $MgSO_4$, $FeSO_4$, $CaCl_2$, $AgNO_3$, $ZnCl_2$, $CuSO_4$, ethylenediaminetetraacetate (EDTA), reduced glutathione, $CoCl_2$, L-cysteine, $(NH_4)_6Mo_7O_{24}$, and phenylmethylsulfonyl fluoride (PMSF)] and a final 10 mM concentration of 2-mercaptoethanol. As a control, buffer A was used in place of the reagent solution. The activity of the treated enzyme was assayed by the same method described above.

pH Dependency of Capsaicin-Hydrolyzing Activity. Buffer A and a 50 mM acetate buffer containing 0.5 mM $CoCl_2$ were used over the pH range from 5.9 to 10.4 and from 4.5 to 6.2, respectively. A 20 μL aliquot of enzyme solution (about 10 μg/mL) was added to 80 μL of a final 130 μM capsaicin solution at different pH values and incubated for 1 h at 37 °C, and the remaining capsaicin concentration was then determined by HPLC as described above.

Thermal Stability of Capsaicin-Hydrolyzing Enzyme. The enzyme solution was incubated in buffer A with or without 0.5 mM $CoCl_2$ for 1 h at 4, 25, 37, 45, 50, 55, and 60 °C, and the remaining activity was then determined as described above.

Optimum Reaction Temperature. To determine the optimum temperature for the enzyme, it was added to a final 130 μM solution of capsaicin and incubated for 1 h at 30, 37, 45, 50, 55, and 60 °C. The residual capsaicin concentration was determined by HPLC as described above.

pH Stability. The enzyme solution was added to 50 mM Tris-HCl buffers at different pH values and incubated at 37 °C for 1 h, after which the residual activity at pH 7.8 was determined.

Substrate Specificity. Various *N*-acetyl-L-amino acids, *N*-lauroyl-L-amino acids, and *N*-lauroyl-L-dipeptides were used as the substrates

for the capsaicin-hydrolyzing enzyme from *S. mobaraensis*. A final 15 mM *N*-acetyl-L-amino acid was hydrolyzed at 37 °C for 1−3 h, and the resulting L-amino acid was assayed by the ninhydrin method (*13*). The hydrolytic activity toward a final 2 mM solution of *N*-lauroyl-L-amino acid was measured by conducting a reaction at 37 °C for 1−2 h, and the resulting L-amino acid was assayed by the ninhydrin method. Hydrolysis toward a final 0.4 mM concentration of *N*-lauroyl-L-dipeptides was carried out at 37 °C for 1−2 h, and the remaining substrate concentration was determined by HPLC at 210 nm, in which the elution was performed by changing the acetonitrile concentration from 40 to 50% with 0.075% phosphoric acid as a mobile phase at a flow rate of 0.8 mL/min.

Synthesis of Capsaicin Derivatives. The synthesis was typically conducted in aqueous/organic biphasic systems composed of 4 mL of organic solvent and 1 mL of buffer D including 10 units of purified enzyme with vigorous magnetic stirring in a glass vial at 37 °C. At appropriate times, a 50 μL aliquot of the organic phase and a 12.5 μL aliquot of the aqueous phase were withdrawn and the concentration of product in the organic phase was determined by HPLC at 280 nm, in which the elution was performed using 65−90% (v/v) acetonitrile solution containing 0.075% phosphoric acid as the mobile phase at a flow rate of 0.8 mL/min at room temperature. In the synthesis of lauroyl vanillylamide, the effects of the volume ratio, pH of the aqueous phase, and substrate concentrations were examined. Various capsaicin derivatives, octanoyl vanillylamide, decanoyl vanillylamide, myristoyl vanillylamide, palmitoyl vanillylamide, stearoyl vanillylamide, and oleoyl vanillylamide, were synthesized by reacting 20 mM vanillylamine and 200 mM the corresponding fatty acid in an *n*-hexane or 1-heptanol/buffer D biphasic system at a volume ratio of 4.

Nuclear Magnetic Resonance (NMR) Analysis of Lauroyl Vanillylamide. Lauroyl vanillylamide formed crystals in the reaction mixture and was recovered with a filtration paper followed by washing several times with acidic water (pH 2). The ^{1}H NMR spectrum of the product dissolved in a $CDCl_3$ solution was obtained using a 300 MHz NMR instrument (Ac-300, Bruker BioSpin GmbH, Karlsruhe, Germany). The chemical shifts are given in δ (ppm) values using tetramethylsilane ($\delta = 0$ ppm) as an internal standard.

Synthesis of *N*-Lauroyl-L-Amino Acids and *N*-Lauroyl-Peptides. *N*-Lauroyl-L-amino acids and *N*-lauroyl-peptides were, respectively, synthesized from 200 mM amino acid/100 mM dipeptide/66 mM tripeptide and 7.5 mM fatty acid as the substrates. The reaction was started by adding a final 10 units/mL of the enzyme in buffer E with or without 78% (v/v) glycerol with magnetic stirring in a glass vial at 37 °C. At appropriate times, a 25 μL aliquot of the reaction mixture was withdrawn. The product concentration and fatty acid concentration were determined by HPLC with detection at 210 nm, in which the elution was performed using, respectively, 35−60 and 65−90% (v/v) acetonitrile solutions containing 0.075% phosphoric acid as the mobile phase at a flow rate of 0.8 mL/min at room temperature.

Fourier Transform Infrared Spectroscopy (FT-IR) Analysis of *N*-Lauroyl-L-Lysyl-Glycine. The *N*-lauroyl-L-lysyl-glycine formed in the reaction mixture was recovered and washed several times with water. The FT-IR spectra of the samples were measured by an attenuated total reflection method using an FT-IR spectrometer (Herschel 480, JASCO Co., Tokyo).

Statistical Analysis. The experimental data were expressed as the means ± SD of values obtained from duplicate or triplicate measurements.

RESULTS AND DISCUSSION

Preparation of the Capsaicin-Hydrolyzing Enzyme. In the course of our investigations of the enzymatic synthesis of capsaicin derivatives, the capsaicin-hydrolyzing activity was occasionally detected in the broth of *S. mobaraensis* NBRC13819. Therefore, in a preliminary experiment, we tested 21 strains of *Streptomyces* sp. listed in **Table 1**, in addition to *S. mobaraensis* NBRC13819 for the presence of a secreted capsaicin-hydrolyzing enzyme. As a result, we detected capsaicin-hydrolyzing activity only in the supernatant of *S. luteoreticuli* NBRC13422 with an activity similar to that for *S. mobaraensis* NBRC13819. Because *S. luteoreticuli* is currently considered a synonym of *S. mobaraensis* by the ATCC, it was thus considered as belonging to the last species. In a subsequent study, we purified the enzyme from the culture broth of *S. mobaraensis* NBRC13819 (hereafter abbreviated as *S. mobaraensis*).

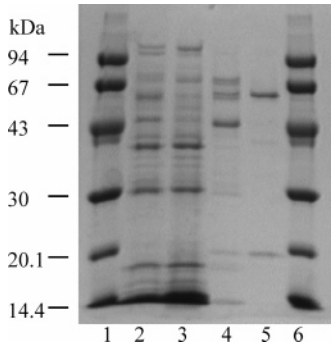

Figure 1. SDS–PAGE of the protein fractions obtained in various purification steps. Lanes 1 and 6, marker proteins; lane 2, culture filtrate; lane 3, 60% ammonium sulfate precipitate; lane 4, the active fraction from CM Sephadex C-50 column chromatography; and lane 5, that from hydroxyapatite column chromatography. As marker proteins, phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used.

Purification of the Enzyme from *S. mobararensis*. The enzyme from *S. mobaraensis* was successively fractionated by column chromatographies on CM Sephadex C-50 gels and hydroxyapatite gels after precipitation with ammonium sulfate. In the CM Sephadex C-50 gel column chromatography, the fractions showing capsaicin-hydrolyzing activity were eluted as a single peak at a NaCl concentration of 400 mM, and in the hydroxyapatite gel chromatography, the enzyme was eluted with 300 mM phosphate buffer. The active fractions recovered from the hydroxyapatite column gave a single band with an estimated molecular mass of around 75 kDa by electrophoresis on a 5−20% polyacrylamide nondenatured gradient gel (data not shown). SDS−PAGE showed the presence of two dissimilar subunits (61 and 19 kDa) as shown in **Figure 1**, indicating that the enzyme is a heterodimeric protein. The two subunits were separated by gel filtration HPLC in the presence of 2 M guanidine hydrochloride. Fractions containing a large subunit, a small subunit, and both fractions were, respectively, dialyzed against buffer A to remove guanidine hydrochloride. The dialyzed three enzyme solutions were assayed for capsaicin-hydrolyzing activity as described previously. Only a mixture containing both subunits showed the activity. These findings indicate that the heterodimeric protein would express the enzymatic activity in this form.

Table 2 summarizes the purification results for each step. The specific activity of the purified enzyme was 210 units/mg with a purification fold higher than 1300.

Although enzymes that show capsaicin hydrolytic activity were reported previously, their activities were very low as compared to that isolated in this study. Van den Heuvel et al. reported that penicillin G acylase from *Escherichia coli* catalyzes the hydrolysis of capsaicin with a specific activity of 3.5×10^{-6} mM/min/mg (*14*). Kawada et al. reported that a capsaicin-hydrolyzing enzyme is distributed in various organs of the rat, including the liver and kidney (*15*). Oi et al. reported on an enzyme from the livers of the chicken, hog, and cow as well as the rat and pointed out that the enzyme is involved in the initial step of capsaicin metabolism (*16*). However, the specific activity of the crude capsaicin-hydrolyzing enzyme from chicken liver

Table 2. Summary of the Purification of a Capsaicin-Hydrolyzing Enzyme from *S. mobaraensis*

purification step	protein (mg)	total activity (units[a])	yield (%)	specific activity (units[a]/mg)	purification (-fold)
culture filtrate	12600	2016	100	0.16	1
ammonium sulfate	2632	806	40	0.31	1.9
CM-SephadexC-50	30.4	762	38	25	157
hydroxyapatite	2.1	440	18	210	1300

[a] One unit is defined as the amount of enzyme required to hydrolyze 1 μmol of capsaicin in 50 mM Tris-HCl buffer, pH 7.8, in 1 h at 37 °C.

was only around 6.0×10^{-3} units/mg, using the same definition as is used in this study. Park and Lee (*17*) subsequently purified two capsaicin-hydrolyzing enzymes with different molecular masses and isoelectric points from rat liver microsomes. They also pointed out that these enzymes were, in fact, previously identified as carboxyesterases. The specific activity for their purified enzyme with pI 5.6 was only around 2.3 units/mg, which is approximately 1% that of the purified *S. mobaraensis* enzyme.

Optimum pH, Thermal Stability, Optimum Reaction Temperature, and pH Stability of the Purified Enzyme. The effects of some reagents and metal ions on capsaicin-hydrolyzing activity were investigated. The activity was increased by 148 ± 3% by addition of a final concentration of 0.1 mM cobalt ions. On the other hand, the presence of EDTA and 1,10-phenanthroline led to a decrease in activity only by 13 ± 2 and 24 ± 2%, respectively. These findings suggest that cobalt ions are bound strongly to the enzyme. PCMB, DTT, $MgSO_4$, $FeSO_4$, $CaCl_2$, $AgNO_3$, $ZnCl_2$, $CuSO_4$, reduced glutathione, L-cysteine, $(NH_4)_6Mo_7O_{24}$, and PMSF had no appreciable effects.

Figure 2A−D shows optimum pH, thermal stability, optimum reaction temperature, and pH stability of the purified enzyme. The pH optimum for the reaction is around 8 (**Figure 2A**). The enzyme is stable in both the presence and the absence of 0.5 mM Co^{2+} at temperatures below 55 °C, while above 55 °C, the enzyme appears to be more stable in the presence of cobalt ions (**Figure 2B**). Thus, cobalt ions seem to increase not only the activity but also the stability of the enzyme. The optimum reaction temperature for a 1 h hydrolysis of capsaicin is approximately 55 °C (**Figure 2C**). The enzyme is stable in the pH range of 5−8 in 50 mM Tris-HCl buffer in the presence of 0.5 mM Co^{2+} at 37 °C (**Figure 2D**). Further experiments are necessary for characterization of cobalt ion binding to the enzyme.

Substrate Specificity. Because the enzyme isolated in this study catalyzes the hydrolysis of the amide bond of capsaicin, we investigated its specificity with respect to various *N*-fatty-acyl-L-amino acids and *N*-fatty-acyl-L-dipeptides. **Table 3** summarizes the substrate specificity for *N*-fatty-acyl-L-amino acids and *N*-fatty-acyl-L-dipeptides in terms of specific activity.

As shown in **Table 3**, the enzyme shows a wide substrate specificity toward *N*-lauroyl-L-amino acids with a specific activity similar to that toward capsaicin. However, the enzyme does not show any activity toward *N*-acetyl-L-amino acids, indicating that the enzyme is classified as a type of long chain acyl aminoacylase. Aminoacylases showing such a wide substrate specificity have not been reported to date (*18*−*21*). Furthermore, the enzyme shows deacylation activity toward *N*-lauroyl-L-dipeptides, although it has no peptidase activity. Such its substrate specificity is unique since carboxypeptidase G_3 from *Pseudomonas* sp. (*20*) and some aminoacylases (*22*) show both deacylation and peptidase activities.

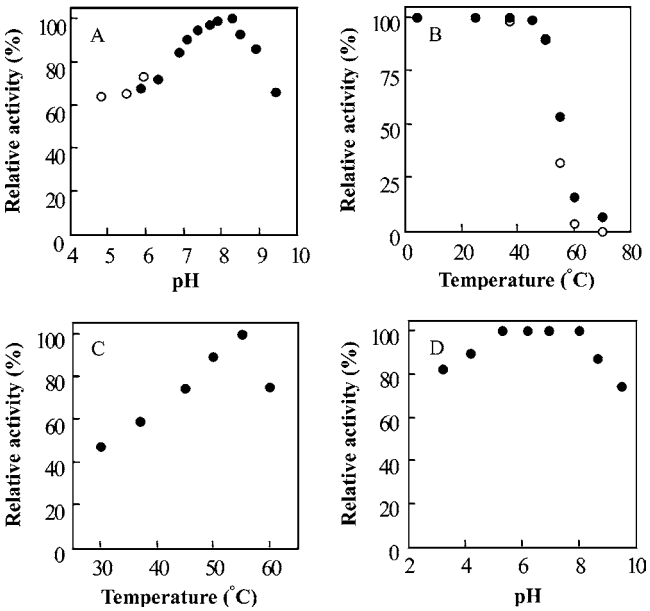

Figure 2. Optimum pH, thermal stability, optimum reaction temperature, and pH stability of the capsaicin-hydrolyzing enzyme from *S. mobaraensis*. (**A**) pH dependency for the capsaicin hydrolysis activity at 37 °C. Fifty millimolar Tris-HCl buffer (●) and 50 mM acetate buffer (○) were, respectively, used in the pH range from 5.9 to 10.4 and that from 4.5 to 6.2. (**B**) Remaining activity of the enzyme after incubation at 4–60 °C for 1 h in 50 mM Tris-HCl, pH 7.8, in the presence of 0.5 mM $CoCl_2$ (●) and in the absence of 0.5 mM $CoCl_2$ (○). (**C**) Optimum reaction temperature for hydrolysis of capsaicin at pH 7.8. (**D**) pH Stability at 37 °C for 1 h.

Synthesis of Capsaicin Derivatives. Capsaicin derivatives were synthesized in an aqueous/organic biphasic system that permits the equilibrium of reaction to be shifted toward synthesis (*8*, *9*). *n*-Hexane was selected as the organic solvent for the reaction. We first examined the effect of reaction conditions on the synthesis of lauroyl vanillylamide from a final concentration of 200 mM lauric acid and 10 mM vanillylamine as the substrates. The optimum volume ratio of organic phase to aqueous phase was approximately 4 (data not shown). The optimum pH of the aqueous phase was 6.9. Thus, the synthetic reaction was carried out by adjusting the pH of the aqueous phase to 6.9 during the course of the reaction. When the lauric acid concentration was increased to 200 mM or higher at a constant 20 mM vanillylamine, the yield could be increased to around 60%. At 200 mM lauric acid, the yield was highest in the vanillylamine concentration range of 10−20 mM. When the synthetic reaction was conducted using 200 mM lauric acid and 20 mM vanillylamine as the substrates in the biphasic reaction system with the volume ratio of 4, the product precipitated as fine needlelike crystals in the *n*-hexane phase. The resulting crystals were washed with *n*-hexane and then subjected to a ^1H NMR analysis. The ^1H NMR spectrum (300 MHz, $CDCl_3$) of the product is shown in **Table 4**. Here, the ^1H NMR spectrum was assigned using spectral data for myristoyl vanillylamide and lauric acid reported in a literature (*23*, *24*).

We also synthesized *N*-fatty-acyl vanillylamides from various fatty acids (200 mM) and vanillylamine (20 mM) using a final 2 units/mL of enzyme in 5 mL of an *n*-hexane/buffer D biphasic system with the volume ratio of 4. The reaction was conducted with magnetic stirring in a glass vial at 37 °C for 4 days. Here, the synthetic yield was calculated on the basis of the amount of vanillylamine used as the substrate. The yield of decanoyl vanillylamide was 81 ± 5%. Myristoyl vanillylamide precipitated in a manner similar to lauroyl vanillylamide as described

Table 3. Substrate Specificity of the Capsaicin-Hydrolyzing Enzyme from *S. mobaraensis*

substrate	specific activity (units[a]/mg)	substrate	specific activity (units[a]/mg)
capsaicin	210 ± 7	*N*-lauroyl-L-histidine	118 ± 3
N-acetyl-L-glutamic acid	0	*N*-lauroyl-L-isoleucine	90 ± 6
N-acetyl-L-glutamine	0	*N*α-lauroyl-L-leucine	129 ± 3
N-acetyl-L-glycine	0	*N*α-lauroyl-L-lysine	244 ± 7
*N*α-acetyl-L-lysine	0	*N*-lauroyl-L-methionine	177 ± 12
*N*ε-acetyl-L-lysine	0	*N*-lauroyl-L-phenylalanine	25 ± 3
N-acetyl-L-methionine	0	*N*-lauroyl-L-serine	295 ± 12
N-lauroyl-L-alanine	236 ± 17	*N*-lauroyl-L-threonine	230 ± 14
N-lauroyl-L-arginine	194 ± 2	*N*-lauroyl-L-tryptophan	22 ± 3
N-lauroyl-L-asparagine	227 ± 23	*N*-lauroyl-L-tyrosine	145 ± 10
N-lauroyl-L-aspartic acid	138 ± 13	*N*-lauroyl-L-valine	87 ± 7
N-lauroyl-L-glutamic acid	196 ± 7	*N*-lauroyl-L-glutaminyl-glycine	110
N-lauroyl-L-glutamine	221 ± 17	*N*-lauroyl-L-glutamyl-L-lysine	70
N-lauroyl-glycine	222 ± 22	*N*-lauroyl-L-glutamyl-glycine	90

[a] One unit of the enzyme activity is defined as the amount of the enzyme required to hydrolyze 1 μmol of substrate in 1 h at 37 °C at pH 7.8.

Table 4. ^{1}H NMR of Lauroyl Vanillylamide

position	δ_H
CH_3	0.88 (t, 3H, J = 6.5 Hz)
$CH_2(CH_2)_8CH_3$	1.26 (m, 16H)
$COCH_2CH_2$	1.65 (m, 2H)
$NHCOCH_2$	2.20 (t, 2H, J = 7.5 Hz)
OCH_3	3.88 (s, 3H)
$ArCH_2NH$	4.36 (d, 2H)
OH, NH	5.68 (2H, br.s)
aryl-H	6.76 (dd, 1H, J = 8.0, 1.8 Hz)
	6.81 (d, 1H, J = 1.8 Hz)
	6.87 (d, 1H, J = 8.0 Hz)

above, but the yield was only 15 ± 1%. This suggests that the reaction had not reached equilibrium because of the lower solubility of myristic acid, as compared to that of lauric acid. The yields of palmitoyl vanillylamide, stearoyl vanillylamide, and oleoyl vanillylamide were only 0.5% or lower after a 4 day reaction (data not shown). Octanoyl vanillylamide was synthesized in a yield of 27 ± 2% in an *n*-hexane/buffer D biphasic system. The yield was increased to 46 ± 3% in a 1-heptanol/buffer D biphasic system probably because of higher partition of the product in 1-heptanol.

Synthesis of *N*-Lauroyl-L-Amino Acids and *N*-Lauroyl-Peptides. As shown previously, the capsaicin-hydrolyzing enzyme from *S. mobaraensis* catalyzes the deacylation of *N*-lauroyl-L-amino acids and *N*-lauroyl-L-dipeptides. We thus attempted to synthesize *N*-lauroyl-L-amino acids in buffer E containing 78% (v/v) glycerol to shift the equilibrium in a manner similar to the syntheses of *N*α-lauroyl-L-amino acids reported previously (*25*). The synthetic reactions were conducted for 2 days at 37 °C, using 200 mM amino acids and 7.5 mM lauric acid as the substrates, in which the final enzyme concentration was 10 units/mL. As shown in **Figure 3**, relatively high synthetic yields were obtained when L-lysine, L-arginine, and L-cysteine were used as the amino acid. When L-lysine was used, both *N*α- and *N*ε-lauroyl-L-lysines were obtained in yields of 5 ± 1 and 44 ± 5%, respectively, as shown in **Figure 3**. The fact that the condensation reaction shows a preference for the ε-amino group of L-lysine can be attributed to the much lower solubility of *N*ε-lauroyl-L-lysine.

The synthetic reaction was conducted using various dipeptides including L-lysine and lauric acid as the substrates in buffer E with or without 78% (v/v) glycerol. Even in the absence of glycerol, *N*ε-lauroyl-dipeptides are efficiently synthesized without the formation of *N*α-lauroyl-dipeptides, as shown in **Figure 4**, in which the synthetic rate and the equilibrium yield are higher than that for the reaction mixture containing glycerol.

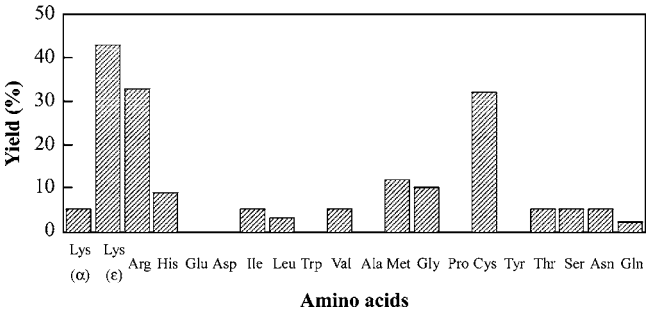

Figure 3. Synthetic yields of various *N*-lauroyl-L-amino acids. The reaction was conducted using 7.5 mM lauric acid, 200 mM amino acids, and 10 units/mL of the enzyme for 2 days.

The position of *N*-acylation was identified by FT-IR analysis using *N*-lauroyl-L-lysyl-glycine as a model substance. An NMR analysis was unsuccessful because the product is sparingly soluble in any solvent. **Figure 5** shows an IR absorption spectra for *N*ε-lauroyl-L-lysyl-glycine, *N*ε-lauroyl-L-lysine, and *N*α-lauroyl-L-lysine. In the case of *N*α-lauroyl-L-lysine, the C−O stretching band is detected at around 1600 cm^{-1} while that for *N*ε-lauroyl-L-lysine is at 1650 cm^{-1}. *N*ε-lauroyl-L-lysyl-glycine shows a band at 1650 cm^{-1} in the C−O stretching region, similar to *N*ε-lauroyl-L-lysine, indicating that the *N*-acylation occurs at the position of the ε-amino group.

In addition to the *N*-lauroyl-dipeptides as shown above, *N*-lauroyl-diglycine, *N*-lauroyl-triglycine, *N*-lauroyl-tri-L-alanine, and *N*-lauroyl-L-leucyl-diglycine were synthesized in buffer E containing 78% (v/v) glycerol (data not shown), which would encourage continuing further study.

In this study, we purified and characterized a novel capsaicin-hydrolyzing enzyme from *S. mobaraensis* with an extremely high specific activity, as compared with previously reported enzymes. The enzyme isolated in this study appears to be the first microbial enzyme that efficiently hydrolyzes capsaicin. It was also found that the enzyme catalyzed the efficient deacylation of *N*-lauroyl-L-amino acids and *N*-lauroyl-peptides in addition to the hydrolysis of capsaicin. Thus, the *S. mobaraensis* enzyme could principally be categorized as an acylase. The enzyme also catalyzed the syntheses of capsaicin derivatives (*N*-fatty-acyl vanillylamide), *N*-fatty-acyl-L-amino acids, and *N*-fatty-acyl-peptides in an aqueous/organic biphasic system and

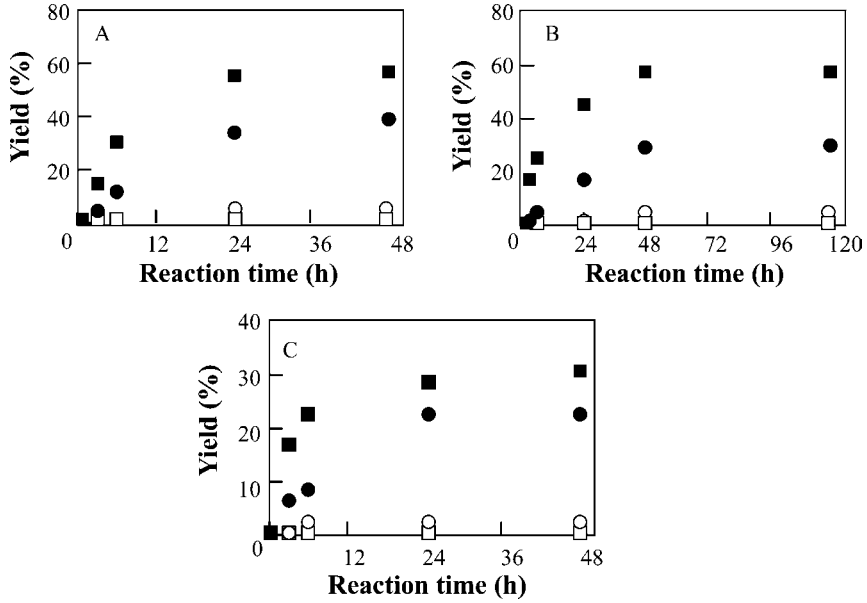

Figure 4. Comparison of the courses for the syntheses of *N*α- and *N*ϵ-lauroyl-L-lysyl-glycines (**A**), *N*α- and *N*ϵ-lauroyl-L-lysyl-L-isoleucines (**B**), and *N*α- and *N*ϵ-lauroyl-glycyl-L-lysines (**C**). *N*α-Lauroyl-dipeptides were synthesized from 100 mM dipeptides and 7.5 mM lauric acid in buffer E with 78% glycerol (○) and without glycerol (□). *N*ϵ-Lauroyl-dipeptides were synthesized in buffer E with 78% glycerol (●) and without glycerol (■).

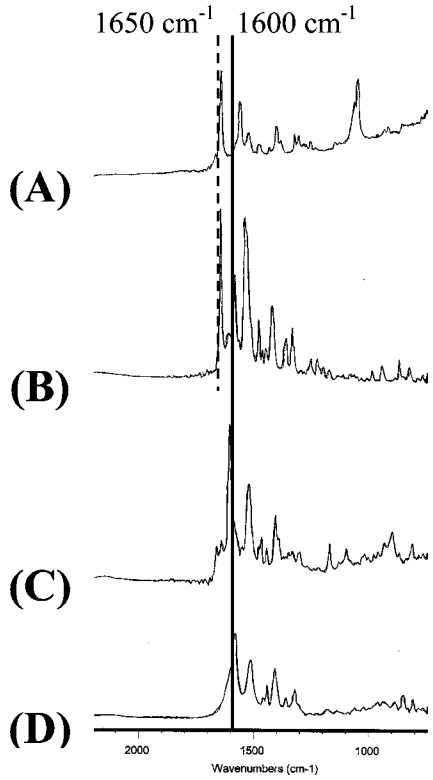

Figure 5. IR spectra of *N*-lauroyl-L-lysyl-glycine (**A**), *N*ϵ-lauroyl-L-lysine (**B**), *N*α-lauroyl-L-lysine (**C**), and L-lysine (**D**). The positions for the wavenumbers at 1600 and 1650 cm^{-1} are shown by solid and dotted lines, respectively.

in an aqueous buffer solution with and without glycerol. The *N*-acylation in the synthesis of *N*-fatty-acyl-dipeptides in the case of lysine occurred preferentially at the ϵ-amino group. Thus, the *S. mobaraensis* enzyme could be potentially utilized in the efficient syntheses of various useful *N*-fatty-acyl compounds because of its high specific activity and wide substrate specificity. A gene analysis of the *S. mobaraensis* enzyme is currently underway, which could enable one to produce in a large scale.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate polyaclylamide gel; CBB R-250, Coomassie Brilliant Blue R-250; PCMB, *p*-chloromercuribenzoic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethylsulfonyl fluoride; NMR, nuclear magnetic resonance; FT-IR, Fourier transform infrared spectroscopy.

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