Biosci. Biotechnol. Biochem., **69** (10), 1914–1922, 2005

Purification and Characterization of a Novel Aminoacylase from *Streptomyces mobaraensis*

Mayuko KOREISHI,[1] Fumiaki ASAYAMA,[1] Hiroyuki IMANAKA,[1] Koreyoshi IMAMURA,[1] Megumi KADOTA,[2] Takuo TSUNO,[2] and Kazuhiro NAKANISHI[1,†]

[1]*Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, 3-1-1 Tsushima-naka, Okayama 700-8530, Japan*

[2]*Tsuno Food Industrial Co., Ltd., 94 Shinden, Katuragi, Ito, Wakayama 649-7194, Japan*

Received May 9, 2005; Accepted June 27, 2005

A novel aminoacylase was purified to homogeneity from culture broth of *Streptomyces mobaraensis*, as evidenced by SDS–polyacrylamide gel electrophoresis (PAGE). The enzyme was a monomer with an approximate molecular mass of 100 kDa. The purified enzyme was inhibited by the presence of 1,10-phenanthroline and activated by the addition of Co^{2+}. It was stable at temperatures of up to 60 °C for 1 h at pH 7.2. It showed broad substrate specificity to *N*-acetylated L-amino acids. It catalyzed the hydrolysis of the amide bonds of various *N*-acetylated L-amino acids, except for *Nε*-acetyl-L-lysine and *N*-acetyl-L-proline. Hydrolysis of *N*-acetyl-L-methionine and *N*-acetyl-L-histidine followed Michaelis–Menten kinetics with K_m values of 1.3 ± 0.1 mM and 2.7 ± 0.1 mM respectively. The enzyme also catalyzed the deacetylation of 7-aminocephalosporanic acid (7-ACA) and cephalosporin C. Moreover, feruloyl-amino acids and L-lysine derivatives of ferulic acid derivatives were synthesized in an aqueous buffer using the enzyme.

Key words: aminoacylase; *Streptomyces mobaraensis*; *N*-acetyl-L-amino acid; ferulic acid

Aminoacylase (*N*-acyl-L-amino-acid amidohydrolase, EC 3.5.1.14) is found in a wide variety of sources including animals, plants, and microorganisms. A number of aminoacylases have been purified and characterized to date, from animal tissues,[1–3] plants,[4] molds,[5,6] and bacteria.[7–13] In addition, the genes for enzymes such as those from *Bacillus stearothermophilus*[10] and *Lactococcus lactis* MG1363[11] have recently been analyzed. In practice, aminoacylase has been intensively utilized for the production of L-amino acids, such as L-alanine, L-methionine, and L-phenylalanine from the corresponding *N*-acetyl-D,L-amino acids.[14,15]

In this study, we report on the isolation of a novel aminoacylase from *Streptomyces mobaraensis*, an actinomycete with wide substrate specificity. The enzyme was purified to homogeneity on SDS–PAGE and characterized. In addition, it was used to synthesize feruloylamino acids and lysine derivatives of ferulic acid derivatives, thus confirming the wide substrate specificity of this enzyme.

Materials and Methods

Materials. Beef extract, malt extract, and yeast extract were obtained from Difco Laboratories (Detroit, MI). Polypepton was purchased from Nihon Pharmaceutical (Tokyo). NZ amine (Type A) and soluble starch were purchased from Wako Pure Chemical Industries (Osaka, Japan). CM Sephadex C-50 and Phenyl Sepharose CL-4B were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Hydroxyapatite (Fast Flow Type) was purchased from Wako Pure Chemical Industries. *N*-Acetylated amino acids were purchased either from Wako Pure Chemical Industries or Sigma-Aldrich (St. Louis, MO). *N*-Chloroacetyl-L-phenylalanine and *N*-chloroacetyl-L-tryptophan were purchased from Sigma-Aldrich. *N*-Lauroyl-L-methionine was synthesized by condensing the *N*-hydroxysuccinimide ester of lauric acid with L-methionine by the method of Lapidot *et al.*[16] L-Methionyl-L-phenylalanine, *Z*-L-methionine, and *Z*-L-histidine were purchased from Kokusan Chemical (Tokyo). Glycyl-L-phenylalanine was purchased from the Peptide Institute (Osaka). Cephalosporin C and 7-ACA were purchased from Sigma-Aldrich and Tokyo Kasei Kogyo (Tokyo) respectively. Amino acids were purchased from either Wako Pure Chemical Industries or the Peptide Institute. Ferulic acid, vanillic acid, and homovanillic acid were purchased from Sigma-Aldrich. Cinnamic acid, caffeic acid, phenylacetic acid, and benzoic acid were purchased from Wako Pure Chemical Industries. Hydrocinnamic acid was purchased from Tokyo Kasei Kogyo. All other reagents were of

† To whom correspondence should be addressed. Tel: +81-86-251-8202; Fax: +81-86-251-8264; E-mail: kazuhiro@cc.okayama-u.ac.jp

Abbreviations: PAGE, polyacrylamide gel electrophoresis; 7-ACA, 7-amino-cephalosporanic acid; BSA, bovine serum albumin; CBB R-250, Coomassie Brilliant Blue R-250; PCMB, *p*-chloromercuribenzoic acid; DTT, dithiothreitol

analytical grade and were purchased either from Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan).

Buffers. The following buffers were used in this study: buffer A: 50 mM Tris–HCl buffer, pH 7.5; buffer B: 25 mM Tris–HCl buffer, pH 7.5; buffer C: 30 mM potassium phosphate buffer, pH 7.5; and buffer D: 100 mM Tris–HCl buffer, pH 7.2.

Microorganism and cultivation of cells. S. mobaraensis NBRC (IFO) 13819, a type culture of NBRC (National Institute of Technology and Evaluation Biological Resource Center, Chiba, Japan) was used in this study. The strain was aseptically transferred to an agar plate (4 g yeast extract, 10 g malt extract, 4 g glucose, and 20 g agar in 1,000 ml water, adjusted to pH 7.3) and statically incubated for 7 d at 30 °C. A loopful of the agar culture was then inoculated in a 300-ml shaking flask containing 30 ml of preculture medium (10 g glucose, 10 g dextrin, 5 g NZ amine [Type A], 5 g yeast extract, and 1 g $CaCO_3$ in 1,000 ml water, adjusted to pH 6.5) and incubated at 30 °C with reciprocal shaking at 120 strokes/min. A 1.6-ml aliquot of the preculture was added to a 500-ml shaking flask containing 50 ml of medium (40 g beef extract, 40 g soluble starch, 20 g Polypepton, 20 g $MgSO_4$, and 2 g K_2HPO_4 in 1,000 ml water, adjusted to pH 7.0) for the main culture. The cells were grown by reciprocal shaking at 120 strokes/min at 30 °C for 8 d. After cultivation, the culture broth was recovered by centrifugation (20,000 × *g*) for 30 min at 4 °C.

Enzyme assays. Aminoacylase activity was assayed using *N*-acetyl-L-methionine dissolved in buffer A at a final concentration of 15 mM as the substrate. The reaction was carried out for 20 min at 37 °C, and the L-methionine produced was assayed by the ninhydrin method, as described elsewhere.[17] One unit of aminoacylase activity was defined as the amount of the enzyme required to produce 1 µmol of L-methionine in 1 min at 37 °C at pH 7.5. Protein concentrations were determined using a BCA Protein Assay Reagent Kit (Pierce Chemical, Rockford, IL) with bovine serum albumin (BSA) as a standard.

Purification of the enzyme. All purification procedures were carried out at 4 °C. Ammonium sulfate was first added to the culture supernatant (780 ml) to 60% saturation to precipitate the proteins, including the enzyme. The precipitate recovered by centrifugation at 20,000 × *g* for 30 min was dissolved in 120 ml of buffer B containing 50 mM NaCl, and dialyzed 2 times against 3,000 ml of the same buffer. The dialyzed solution was placed on a CM Sephadex C-50 gel column (1.6 i.d. × 35 cm) equilibrated with the same buffer as was used for the dialysis. Elution was done by a linear increase in the NaCl concentration in buffer B from 50 mM to 500 mM at a flow rate of 0.2 ml/min. Fractions showing aminoacylase activity were collected and concentrated using polyethylene glycol (M.W.: 20,000) through a dialysis membrane. The concentrated enzyme solution was dialyzed against 1,000 ml of buffer C. The resulting enzyme solution was applied to a hydroxyapatite gel column (1.6 i.d. × 15 cm) and eluted by linearly increasing the concentration of potassium phosphate buffer, pH 7.5, from 30 to 300 mM at a flow rate of 0.18 ml/min. The active fractions eluted were collected, concentrated, and dialyzed against 1,000 ml of buffer A containing 700 mM sodium sulfate. The enzyme solution was applied to a Phenyl Sepharose CL-4B gel column (1.6 i.d. × 12 cm) and eluted by a linear decrease in sodium sulfate concentration in buffer A from 700 to 0 mM at a flow rate of 0.25 ml/min. The active fractions were collected and finally dialyzed against 1,000 ml of buffer A.

Native-PAGE and SDS–PAGE. Native-PAGE was carried out using a 5–20% non-denatured gradient gel (Bio-Rad Laboratories, Mini-Protean III Ready Gels J, Hercules, CA) in which acetic acid/β-alanine buffer, pH 4.5, was used as a reservoir buffer according to Reisfeld *et al.*[18] As marker proteins for native-PAGE, glyceraldehyde-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 Chemical. The protein bands were stained with Coomassie Brilliant Blue R-250 (CBB R-250; Sigma-Aldrich). The molecular mass of the enzyme was determined from a calibration curve prepared from the mobilities of the marker proteins *versus* the logarithms of their molecular weights.

SDS–PAGE was performed using a 12.5% gel (Bio-Rad Laboratories, Mini-Protean III Ready Gels J) by the method of Laemmli.[19] 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) (Amersham Pharmacia Biotech, LMW Electrophoresis Calibration Kit) were used as standard proteins. The protein bands were stained with CBB R-250.

Effects of reagents on the stability of aminoacylase. A 18-µl aliquot of the enzyme solution (77 µg/ml) was added to either 2 µl of 10 mM of reagent (*p*-chloromercuribenzoic acid [PCMB], iodoacetamide, dithiothreitol [DTT], GSH, L-cysteine, 1,10-phenanthroline, and EDTA) dissolved in buffer A, or 2 µl of 100 mM β-mercaptoethanol in the same buffer, and pre-incubated for 15 min at 37 °C. As a control, 2 µl of buffer A was used in place of the reagent solution. A 1.98 ml aliquot of the *N*-acetyl-L-methionine solution, dissolved in buffer A, was added at a final concentration of 15 mM, and the solution was incubated for 20 min at 37 °C.

Residual enzyme activity was assayed by the method described above.

Effects of metal ions on activity. The enzyme solution was dialyzed against 200 ml of buffer A containing 10 mM 1,10-phenanthroline for 24 h at 4 °C to prepare a metal-free enzyme, followed by dialysis against 200 ml of buffer A for 24 h at 4 °C to remove 1,10-phenanthroline. A 18-µl aliquot of the metal-free enzyme solution (77 µg/ml) thus prepared was then added to 2 µl of 10 mM metal solutions ($MgSO_4$, $FeSO_4$, $CaCl_2$, $AgNO_3$, $ZnSO_4$, $CuSO_4$, $CoCl_2$, $MnSO_4$, $(NH_4)_6Mo_7O_{24}$, and KCl) dissolved in buffer A and pre-incubated for 1 h at 37 °C. As a control, 2 µl of buffer A was used in place of the reagent solution. Enzyme activity was assayed by the method described above.

pH dependencies of activity and stability. In the subsequent experiments, the enzyme solution, dissolved at a concentration of 70 µg/ml in buffer A containing 0.1 mM Co^{2+} (hereafter referred to as purified enzyme solution) was used.

To investigate the pH dependency on enzyme activity, a 20-µl aliquot of purified enzyme solution was added to 1.98 ml of an *N*-acetyl-L-methionine solution, pHs 5.0–9.5, at a final concentration of 15 mM, and incubated for 20 min at 37 °C. The enzyme activity was determined by measuring the concentration of L-methionine formed, as described above.

The pH stability of the enzyme was determined as follows: a 20-µl aliquot of purified enzyme solution was added to 180 µl of 50 mM Tris–HCl buffer, pHs 2.8–12.2, and incubated for 1 h at 37 °C. A 1.8-ml aliquot of *N*-acetyl-L-methionine solution was then added at a final concentration of 15 mM, and the solution was incubated for 20 min at 37 °C. The remaining activity was determined by the method described above.

Optimum reaction temperature and thermal stability. For determination of the optimal reaction temperature, a 5-µl aliquot of purified enzyme solution was added to 495 µl of the *N*-acetyl-L-methionine solution at a final concentration of 15 mM. The reaction mixture was then incubated for 20 min at 4, 25, 37, 50, 60, 70, and 80 °C and the activity subsequently determined.

The thermal stability of the enzyme was investigated. A 5-µl aliquot of purified enzyme solution was added to 95 µl of buffer A and the solution was incubated for 1 h at 4, 25, 37, 50, 60, 70, and 80 °C. The treated enzyme solution was then added to 400 µl of an *N*-acetyl-L-methionine solution at a final concentration of 15 mM, followed by incubation for 20 min at 37 °C, after which residual activity was determined.

Substrate specificity. Hydrolytic activity towards various *N*-acylated amino acids was assayed as described below. A 20-µl aliquot of purified enzyme solution was added to 1.98 ml of a solution of the substrate (15 mM *N*-acetyl-L-amino acids, *N*-acetyl-D-methionine, *N*-chloroacetyl-L-phenylalanine, and *N*-chloroacetyl-L-tryptophan, 1 mM *N*ε-acetyl-L-lysine, and 2 mM *N*-lauroyl-L-methionine). The reaction was allowed to proceed for 20 min at 37 °C, and the concentration of amino acids released in the reaction was determined by the ninhydrin method.[17]

Z-L-Amino acids were also hydrolyzed by the purified enzyme as follows: a 10-µl aliquot of purified enzyme solution was added to 990 µl of solutions of *Z*-L-methionine or *Z*-L-histidine in buffer A at a final concentration of 15 mM, followed by incubation for 2 h at 37 °C. The amount of benzyloxycarbonic acid released was determined by HPLC (Shimadzu, Kyoto) using a YMC-Pack C8 A-202 column (4.6 i.d. × 150 mm, YMC, Kyoto) with detection at 260 nm. A mixture of methanol and water (65:35, v/v) including 0.075% phosphoric acid, pH 3, was used as the mobile phase, at a flow rate of 0.5 ml/min at room temperature.

Activity towards L-methionyl-L-phenylalanine and glycyl-L-phenylalanine was determined by adding a 10-µl aliquot of the purified enzyme solution to 990 µl of a solution of dipeptide in buffer A at a final concentration of 15 mM incubated for 3 and 0.25 h at 37 °C. The amount of L-phenylalanine released was analyzed by HPLC, in which the elution was performed by feeding a mixture of methanol and water (15:85, v/v) including 0.075% phosphoric acid, pH 3, as a mobile phase at a flow rate of 0.5 ml/min, with detection at 260 nm.

Hydrolytic activity towards cephalosporin C and 7-ACA was measured by adding a 10-µl aliquot of the purified enzyme solution to 990 µl of substrate dissolved in buffer A at a final concentration of 2 mM. The reaction was carried out for 10 min at 37 °C. Residual substrate concentration was assayed by HPLC at 260 nm, in which a mixture of methanol and water (20:80, v/v) including 0.075% phosphoric acid, pH 3, was fed as a mobile phase at a flow rate of 0.5 ml/min.

The initial reaction kinetics for the hydrolysis of *N*-acetyl-L-methionine and *N*-acetyl-L-histidine were determined as follows. A 10-µl aliquot of purified enzyme solution was added to 1.99 ml of a substrate solution at different final concentrations of 1–20 mM and incubated at 37 °C. At appropriate times, a 10-µl portion was withdrawn and the concentration of L-amino acid formed was assayed. The initial reaction rates were evaluated from the linear part of the plot.

Synthetic reactions using various amino acids and carboxylic acids. Various ferulic acid derivatives and amino acids were used as substrates for the condensation of feruloylamino acids and L-lysine derivatives of ferulic acid derivatives. Vanillic acid, benzoic acid, homovanillic acid, phenylacetic acid, ferulic acid, caffeic acid, cinnamic acid, and hydrocinnamic acid were used as ferulic acid derivatives. The condensation reaction was typically conducted in buffer D with or without glycerol. To investigate the optimum pH and substrate concen-

tration for the synthesis of *Nα*-feruloyl-L-lysine, a reaction was run in 100 mM Tris–HCl buffer, in a pH range of 5.3–8.0. The reaction was carried out with vigorous magnetic stirring in a glass vial at 37 °C. At appropriate times, a 25-μl aliquot of the reaction solution was withdrawn for product assay. Product concentration was assayed by HPLC with detection at 322 nm, in which a mixture of methanol and water (45:55, v/v) adjusted to pH 3.0 with 0.075% phosphoric acid was fed as the mobile phase at a flow rate of 0.5 ml/min. The yield was calculated on the basis of the initial molar concentration of ferulic acid.

Evaluation of equilibrium constants. The equilibrium constant of the synthetic reaction for *Nα*-feruloyl-L-lysine was calculated using equation (1):

$$K = [N\alpha\text{-feruloyl-L-lysine}]_{eq}[H_2O] / [\text{ferulic acid}]_{eq}[\text{L-lysine}]_{eq} \quad (1)$$

The term $[\ ]_{eq}$ denotes the equilibrium concentration of the components. The concentration of *Nα*-feruloyl-L-lysine was determined by HPLC, as described above.

NMR analysis of Nα-feruloyl-L-lysine. A synthetic reaction mixture from ferulic acid and L-lysine was first adjusted to pH 2.5 to stop the reaction and the ferulic acid was removed by extraction with ethyl acetate. The aqueous phase, containing the product (*Nα*-feruloyl-L-lysine), was extracted into *n*-butanol and dried *in vacuo* to give a dried powder. The powder was dissolved in D_2O and a ^{1}H-NMR spectrum was obtained using a 300 MHz NMR instrument (Ac-300, Bruker BioSpin GmbH, Karlsruhe, Germany). Chemical shifts are reported in δ (ppm) values using TMS ($\delta = 0$ ppm) as an internal standard.

Results and Discussion

Purification of the enzyme

The enzyme was successively fractionated by chromatographies on columns of CM Sephadex C-50 gels, hydroxyapatite gels, and Phenyl Sepharose CL-4B gels. In CM-Sephadex C-50 column chromatography, the fractions showing aminoacylase activity were eluted as a single peak at a NaCl concentration of approximately 250 mM. In hydroxyapatite gel chromatography, the active fractions appeared at a concentration of phosphate buffer of 220 mM. Finally, the enzyme was eluted with 0 mM sodium sulfate in buffer A from a Phenyl Sepharose CL-4B column. The active fraction collected from the final column was found to be homogeneous by SDS–PAGE, as shown in Fig. 1, and had a molecular mass of approximately 100 kDa. Furthermore, electrophoresis on a 5–20% polyacrylamide non-denatured gradient gel also gave a single band with an estimated molecular weight of around 100 kDa, indicating that the enzyme is monomeric.

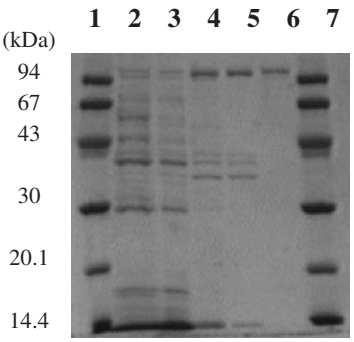

Fig. 1. SDS–PAGE of the Protein Fractions Eluted by Various Column Chromatographies.

Lanes 1 and 7, marker proteins; lane 2, culture filtrate; lane 3, 60% ammonium sulfate precipitate; lane 4, the active fraction from CM Sephadex C-50 column chromatography; lane 5, that from hydroxyapatite column chromatography; lane 6, that from Phenyl Sepharose CL-4B 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.

Table 1. Summary of the Purification of an Aminoacylase from *S. mobaraensis*

Purification step	Protein (mg)	Total activity (units[a])	Yield (%)	Specific activity (units/mg)	Purification (-fold)
Culture filtrate	9438	2497	100	0.26	1.0
Ammonium sulfate	1215	1191	47.7	0.98	3.8
CM-SephadexC-50	25.2	1069	42.8	42	162
Hydroxyapatite	8.4	482	19.3	57	219
Phenyl Sepharose CL-4B	1.5	222	8.9	152[b]	585

[a]One unit was defined as the amount of enzyme required to hydrolyze 1 μmol of *N*-acetyl-L-methionine in 50 mM Tris–HCl buffer, pH 7.5, in 1 min at 37 °C.
[b]The specific activity after the addition of 0.1 mM Co^{2+} was 209 units/mg.

The purification steps for the enzyme are summarized in Table 1. The specific activity of the purified enzyme was determined to be approximately 152 units/mg using 15 mM *N*-acetyl-L-methionine as a substrate with a yield of 9%.

Some properties of the purified enzyme

Table 2 summarizes the effects of various reagents on aminoacylase activity. Activity relative to that measured in the absence of reagents is shown. The fact that 1,10-phenanthroline decreased activity by approximately 60% indicates that the enzyme is a metallo-enzyme. When the enzyme was dialyzed against a solution of 10 mM 1,10-phenanthroline in buffer A overnight, it lost approximately 85% of its activity. However, with the addition of a final concentration of 0.1 mM Co^{2+} to the enzyme solution after dialysis, activity increased by 320%, as shown in Table 3. Thus this enzyme required Co^{2+} for catalytic activity. Subsequent experiments were carried out using an enzyme solution containing a final concentration of 0.1 mM Co^{2+}.

Table 2. Effect of Reagents on Enzyme Activity

Reagents	Concentration[a] (mM)	Relative activity (%)
Control[b]	—	100
PCMB	1	77
Iodoacetamide	1	96
β-Mercaptoethanol	10	100
DTT	1	99
GSH	1	94
L-Cysteine	1	93
1,10-Phenanthroline	1	38
EDTA	1	94

[a]Concentration at first incubation.
[b]Enzyme solution without any reagents present.

Table 3. Effects of Metal Ions on Recovery of Enzyme Activity

Metal ions	Concentration[a] (mM)	Relative activity (%)
Control[b]	—	100
$MgSO_4$	0.1	56
$FeSO_4$	0.1	25
$CaCl_2$	0.1	36
$AgNO_3$	0.1	2
$ZnSO_4$	0.1	142
$CuSO_4$	0.1	20
$CoCl_2$	0.1	321
$MnSO_4$	0.1	110
$(NH_4)_6MO_7O_{24}$	0.1	41
KCl	0.1	117

[a]Concentration at first incubation.
[b]Enzyme solution without any metal ions present.

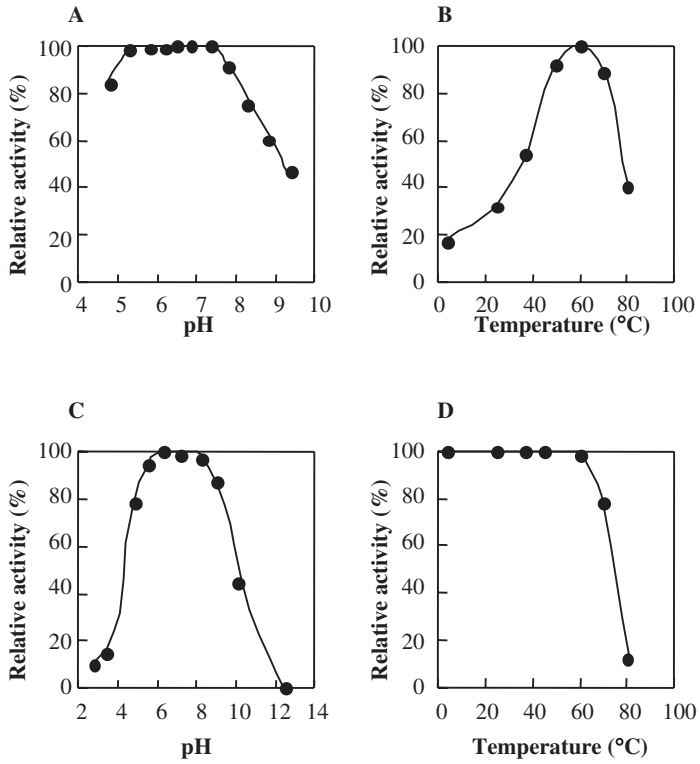

Fig. 2. Characteristics of the Aminoacylase from *S. mobaraensis*.
A, pH dependency of *N*-acetyl-L-methionine hydrolysis activity at 37 °C. B, pH stability of the enzyme incubated at 37 °C for 1 h. C, Thermal stability of the enzyme incubated at different temperatures for 1 h. D, Optimum reaction temperature for hydrolysis of *N*-acetyl-L-methionine at pH 7.5.

Figures 2(A)–(D) show some selected properties of the purified enzyme. The optimum pH for the reaction was in the range of 5.5 to 7.5 at 37 °C (Fig. 2A). The optimum reaction temperature was approximately 60 °C at pH 7.5 (Fig. 2B). The enzyme was stable in a pH range of 5.5 to 8.0 at 37 °C (Fig. 2C) and at temperatures below 60 °C for a 1-h incubation at pH 7.5 (Fig. 2D).

Substrate specificity

Table 4 summarizes substrate specificity with respect to 7-ACA and cephalosporin C as well as various derivatives of amino acids in terms of specific activity. The enzyme from *S. mobaraensis* catalyzed the hydrolysis of most of the *N*α-acetyl-L-amino acids except for *N*-acetyl-L-proline. The enzyme showed high activities, particularly towards *N*-acetylated amino acids with bulky side chains on amino acid residues, such as *N*-acetyl-L-arginine, *N*-acetyl-L-histidine, *N*-acetyl-L-asparagine, *N*-acetyl-L-glutamine, *N*-acetyl-L-leucine, *N*-acetyl-L-methionine, *N*-acetyl-L-phenylalanine, *N*-acetyl-L-tryptophan, and *N*-acetyl-L-tyrosine, while activities for *N*-acetyl-L-cysteine and *N*-acetyl-glycine were

Table 4. Substrate Specificity of the Aminoacylase from *S. mobaraensis*

Substrate	Specific activity (units[a]/mg)	Substrate	Specific activity (units[a]/mg)
N-Acetyl-L-arginine	181	*N*-Acetyl-L-proline	0
N-Acetyl-L-histidine	238	*N*-Acetyl-L-tryptophan	200
Nα-Acetyl-L-lysine	41	*N*-Acetyl-L-tyrosine	158
Nε-Acetyl-L-lysine[b]	0	*N*-Acetyl-L-valine	128
N-Acetyl-L-aspartic acid	38	*N*-Acetyl-D-methionine	0
N-Acetyl-L-glutamic acid	110	*N*-Lauroyl-L-methionine[c]	12
N-Acetyl-L-alanine	124	*N*-Chloroacetyl-L-phenylalanine	90
N-Acetyl-L-asparagine	172	*N*-Chloroacetyl-L-tryptophan	118
N-Acetyl-L-cysteine	15	L-Methionyl-L-phenylalanine	<1
N-Acetyl-L-glutamine	201	Glycyl-L-phenylalanine	65
N-Acetyl-L-glycine	24	*Z*-L-Methionine	1.1
N-Acetyl-L-leucine	188	*Z*-L-Histidine	<1
N-Acetyl-L-methionine	209	7-ACA[c]	35
N-Acetyl-L-phenylalanine	198	Cephalosporin C[c]	8

[a]One unit corresponds to the amount of enzyme required to hydrolyze 1 μmol of substrate in 50 mM Tris–HCl buffer, pH 7.5, in 1 min at 37 °C.
[b]Substrate concentration of 1 mM.
[c]Substrate concentration of 2 mM.

Table 5. Substrate Specificity of Aminoacylases from Various Sources

Enzyme	Hog kidney[1)]	*Aspergillus oryzae*[5)]	*Alcaligenes denitrificans* DA181[7)]	*Bacillus stearothermo -philus*[10)]	*Lactococcus lactis* MG1363[11)]	*Streptomyces mobaraensis*
Substrate	Relative activity (%)[a]					
Ac-Arg	2	—	0	<1	<1	76
Ac-His	<1	—	0	34	<1	100
Nα-Ac-Lys	—	—	—	—	26	17
Nε-Ac-Lys	—	—	—	—	<1	0
Ac-Asp	<1	7	2	<1	32	16
Ac-Glu	13	26	1	2	31	46
Ac-Ala	13	50	74	100	100	52
Ac-Asn	—	—	3	—	—	72
Ac-Cys	—	—	—	—	23	6
Ac-Gln	—	—	—	—	—	84
Ac-Gly	—	—	—	44	—	10
Ac-Ile	2	—	—	—	46	—
Ac-Leu	22	21	4	38	50	79
Ac-Met	100	69	23	11	21	88
Ac-Phe	<1	100	40	51	80	83
Ac-Pro	<1	—	—	—	30	0
Ac-Trp	<1	51	11	1	72	84
Ac-Tyr	—	—	42	90	76	66
Ac-Val	7	—	100	44	56	54

[a]The highest activity towards the particular substrate obtained for each enzyme was taken to be 100%.

low. It had very low activity for *Nε*-acetyl-L-lysine and *N*-acetyl-D-methionine, and its hydrolytic activity with respect to *N*-lauroyl-L-methionine was very low in comparison with that towards *N*-acetyl-L-methionine, indicating that the enzyme favors a short-chain acyl moiety over a long-chain one for *N*-acyl moiety. The hydrolytic activity for *N*-chloroacetyl-L-amino acids was lower than that for the corresponding *N*-acetyl-L-amino acids. Although the enzyme showed a relatively high activity for the hydrolysis of glycyl-L-phenylalanine, it was unable to hydrolyze L-methionyl-L-phenylalanine, *Z*-L-methionine, or *Z*-L-histidine at all. These results indicate that the enzyme favors a substrate with a small acyl moiety, such as acetyl and glycyl residues.

A number of studies have reported data on the substrate specificity of aminoacylases from various sources.[1–13)] Table 5 shows the substrate specificity of some aminoacylases. As shown in Table 5, aminoacylase from *S. mobaraensis* shows wider substrate specificity than those from the other sources. Aminoacylases from mammalian tissues such as hog kidney[1)] (Table 5) and bovine liver[3)] show a very narrow range of substrate specificity. Aminoacylase from *Aspergillus oryzae* (Table 5), which is produced industrially, shows substrate specificity for *N*-acetyl-L-phenylalanine, *N*-acetyl-L-methionine, *N*-acetyl-L-tryptophan, and *N*-acetyl-L-

alanine, but its specific activity is very low.[5,6] Aminoacylases from *Alcaligenes denitrificans* DA181,[7] *Pseudomonas maltophila* B1,[8] and *B. thermoglucosidius*[9] show high specific activity, but the substrate specificity is narrow. As shown in Table 5, the enzyme from *A. denitrificans* DA181 preferentially hydrolyzes *N*-acetyl-L-alanine and *N*-acetyl-L-valine. Some aminoacylases, such as those from *B. stearothermophilus*,[10] *L. lactis* MG1363,[11] *Pyrococcus horikoshii* OT3,[12] and *Thermococcus litoralis*,[13] have been expressed in recombinant *Escherichia coli* cells. The enzymes from *B. stearothermophilus* and *L. lactis* MG1363 have substrate specificity towards *N*-acetyl-L-amino acids with hydrophobic amino acid residues (Table 5). An aminoacylase from *L. lactis* MG1363 has a relatively wide substrate specificity for *N*-acetyl-L-amino acids with neutral aliphatic and aromatic amino acid residues, while its activity with respect to *N*-acetylated histidine and arginine (Table 5), which are good substrates for the enzyme from *S. mobaraensis* isolated in this study, is negligible. The enzyme from *S. mobaraensis* shows a wider specificity and effectively catalyzes the hydrolysis of most *N*α-acetyl-L-amino acids, as described above. Furthermore, and interestingly, the enzyme from *S. mobaraensis* hydrolyzes 7-ACA and cephalosporin C at ester bonds. These esterification reactions were confirmed by the observation that incubating 7-ACA and cephalosporin C with the enzyme yielded deacetyl 7-ACA and deacetyl cephalosporin C in the reaction mixture, which were detected by HPLC. Although such a deacetylation reaction towards 7-ACA and cephalosporin C is known to be catalyzed by cephalosporin C deacetylase isolated from various sources such as citrus peel, *Cephalosporium acremonium*, *B. subtilis* SHS 0133, and *Rhodotorula glutinis* 38B1,[20–23] no acylases that catalyze the hydrolysis of these substrates have been reported. The findings obtained indicate that the aminoacylase from *S. mobaraensis* is unique and novel in its broader substrate specificity in comparison with previously reported enzymes.

The substrate concentration dependency of the initial rate for the hydrolyses of *N*-acetyl-L-methionine and *N*-acetyl-L-histidine followed Michaelis–Menten kinetics (data not shown). The values of K_m and V_{max} for *N*-acetyl-L-methionine were determined to be 1.3 ± 0.1 mM and 323 ± 2.5 μmol/min/mg respectively, while those for *N*-acetyl-L-histidine were 2.7 ± 0.1 mM and 392 ± 8.6 μmol/min/mg respectively.

Synthetic reaction using aminoacylase from S. mobaraensis

As described above, the aminoacylase from *S. mobaraensis* isolated in this study has broad substrate specificity, in particular towards *N*-acetyl-L-amino acids. Hence we searched for the possibility of hydrolytic activity towards amide substances formed by the condensation of carboxylic acids and amino acids other than *N*-acyl-L-amino acids, but commercially available compounds with amide bonds are few and sometimes difficult to synthesize chemically. Hence, in this study, we attempted to synthesize various amide compounds starting from carboxylic acids and amino acids using the reverse of hydrolysis (the condensation reaction). Ferulic acid and its derivatives were used as carboxylic acids, since these compounds are known to have a variety of biological activities, such as radical-scavenging activity, UV absorption potency, and so on.[24–26] By adding amino acids to ferulic acid and its derivatives, the solubility and stability as well as biological activities would be increased. The equilibrium for the condensation reaction is thermodynamically shifted in the direction of hydrolysis. Hence, we first conducted a synthetic reaction in buffer D containing 70% glycerol to shift the equilibrium, similarly to the strategy used in the synthesis of *N*-lauroyl-L-amino acids reported previously.[27] Condensation products were prepared by reacting various L-amino acids (0.5 M) with ferulic acid (0.01 M) for 6 d, a sufficiently long period at 37 °C using the purified enzyme (2.5 units/ml). As shown in Fig. 3, the aminoacylase from *S. mobaraensis* adds most amino acids, except for L-proline and L-tyrosine, to ferulic acid, and particularly high synthetic yields were obtained for L-phenylalanine, L-methionine, L-lysine, and L-arginine. But no products were detected, using an aminoacylase

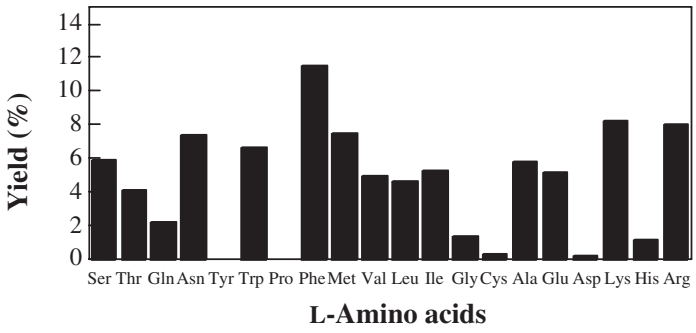

Fig. 3. Synthesis Reaction Using Various Amino Acids and Ferulic Acid.

The condensation reaction was conducted in 100 mM Tris–HCl buffer, pH 7.2, containing 70% glycerol, 0.01 M ferulic acid, and 0.5 M various amino acids. The reaction was carried out for 6 d at 37 °C.

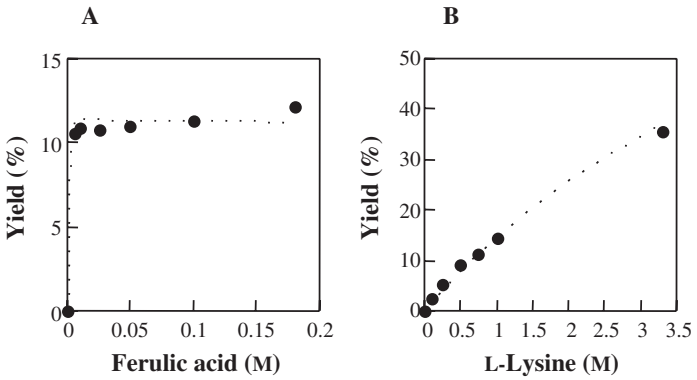

Fig. 4. Yields of *Nα*-Feruloyl-L-lysine under Various Conditions.

A, Effect of ferulic acid concentration on the yield of *Nα*-feruloyl-L-lysine. A reaction mixture containing 0.005–0.18 M ferulic acid, 0.75 M L-lysine, and 2.5 units/ml enzyme in 100 mM Tris–HCl buffer, pH 7.2, was incubated for 4 d at 37 °C with stirring. The dotted line represents the yield of *Nα*-feruloyl-L-lysine calculated using the equilibrium constant, 9.4. B, Effect of L-lysine concentration on the yield of *Nα*-feruloyl-L-lysine. A reaction mixture containing 0.01 M ferulic acid, 0.1–3.3 M L-lysine, and 2.5 units/ml enzyme in 100 mM Tris–HCl buffer, pH 7.2, was incubated for 4 d at 37 °C with stirring. The dotted line represents the yield of *Nα*-feruloyl-L-lysine calculated using the equilibrium constant, 9.4.

from pig kidney at a final concentration of 2.5 units/ml.

The selective acylation of one of two amine groups of L-lysine alone with carboxylic acid by the chemical method is usually difficult.[28] Thus, in this study, the reaction conditions for synthesis of *Nα*-feruloyl-L-lysine were optimized. The yields after 5 h of reaction were increased remarkably by decreasing the glycerol concentration (data not shown). The yield obtained in buffer D without glycerol was about 15 times higher than that for the synthetic reaction using buffer D containing 70% glycerol. Hydrophilic solvents such as glycerol tend to decrease the internal flexibility of the enzyme due to lowering of the dielectric constant of the medium, and remove water molecules around the surface of the enzyme molecule, which lowers enzyme activity and stability respectively.[27,29]

As shown in Fig. 4A, the yield for *Nα*-feruloyl-L-lysine was almost unaffected by increasing the ferulic acid concentration in the range of 0.005 to 0.18 M, while it increased with increasing concentrations of L-lysine, as shown in Fig. 4B because the L-lysine concentration was varied up to a maximum of 3.3 M. The yield, when 3.3 M L-lysine and 0.1 M ferulic acid was used, was approximately 36% after 4 d. The equilibrium constant of the synthetic reaction for *Nα*-feruloyl-L-lysine was calculated from equation (1) using the experimental data and estimated to be, on average, approximately 9.4. The yields calculated using the equilibrium constant determined are in good agreement with the experimental data, as shown in Figs. 4A and B, indicating that the synthetic reaction was controlled by the equilibrium. The optimum pH for the initial rate for the synthesis of *Nα*-feruloyl-L-lysine was found to be 6.5 (data not shown), under which condition, the yield reached about 35% after 2 d, using 3.3 M L-lysine, 0.1 M ferulic acid, and 2.5 units/ml of purified enzyme. The product was obtained as a white powder, as described in "Materials and Methods", and was identified by ^{1}H-NMR and ^{1}H–^{1}H COSY measurements. NMR δ (D_2O): 7.34 (1H, doublet, $J = 15.6$ Hz, Ar–C*H*=CH), 7.02 (1H, multiplet, Ar*H*), 7.00 (1H, multiplet, Ar*H*), 6.85 (1H, doublet, $J = 7.8$ Hz, Ar*H*), 6.43 (1H, d, $J = 15.6$ Hz, Ar–CH=C*H*), 3.83 (3H, singlet, OCH_3), 4.40 (1H, double doublet, $J = 5.4$, 8.4 Hz, NH–C*H*(COOH)–CH_2), 3.04 (2H, triplet, $J = 7.5$ Hz, CH_2–NH_2), 1.95 (2H, multiplet, CH–CH_2–CH_2), 1.76 (2H, multiplet, –CH_2–CH_2–NH_2), 1.52 (2H, multiplet, CH–CH_2–CH_2–CH_2). The ^{1}H-NMR spectrum was assigned on the basis of data for ferulic acid and lysine reported in the literature.[26,30]

Table 6. Synthesis of Ferulic Acid Derivatives and L-Lysine

Compounds	Yield[a] (%)
Benzoic acid	5.0
Vanillic acid	4.0
Phenylacetic acid	9.1
Homovanillic acid	6.6
Hydrocinnamic acid	43.2
Cinnamic acid	32.0
Ferulic acid	35.5
Caffeic acid	24.6

[a]Yields obtained after 48 h of reaction.

Furthermore, several L-lysine derivatives of ferulic acid derivatives were synthesized from ferulic acid derivatives, *viz.*, vanillic acid, benzoic acid, homovanillic acid, phenylacetic acid, caffeic acid, cinnamic acid, and hydrocinnamic acid, under the optimum conditions determined above for the synthesis of *Nα*-feruloyl-L-lysine. The yields for various condensation products after a 48-h reaction are shown in Table 6. When compounds having a structural formula similar to ferulic acid, such as caffeic acid, cinnamic acid, or hydrocinnamic acid, were used as the substrate, condensation products were obtained in yields of 20–45%. But the yields were much lower when vanillic acid, benzoic acid, homovanillic acid, and phenylacetic acid were used.

In this study, we report on the characterization of a novel aminoacylase from *S. mobaraensis* with high thermal stability and wide substrate specificity. Further studies on its genetic properties and structural analysis are required, and are currently underway.

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