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Purification, Characterization, Molecular Cloning, and Expression of a New Aminoacylase from *Streptomyces mobaraensis* That Can Hydrolyze *N*-(Middle/Long)-chain-fatty-acyl-L-amino Acids as Well as *N*-Short-chain-acyl-L-amino acids

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We report here on the purification, characterization, molecular cloning, and expression of a new aminoacylase, initially isolated from the supernatant of *Streptomyces mobaraensis* (*Sm*-AA). Purified wild-type *Sm*-AA was found to be a monomeric protein with a molecular mass of 55 kDa. The cloned gene of *Sm*-AA contained an ORF of 1,383 bp, encoding a polypeptide of 460 amino acids. A BLAST search revealed that *Sm*-AA belongs to the peptidase M20 family, with identities to a hypothetical protein from *Streptomyces pristinaespiralis*, a putative peptidase from *Streptomyces avermitilis*, peptidase M20 from *Frankia* sp., succinyl-diaminopimelate desuccinylase from *Hemophilus influenzae*, and aminoacylase-1 from porcine kidney at 89, 88, 67, 29, and 25% respectively. The *Sm*-AA gene was subcloned into an expression vector, pSH19, and was expressed in *Streptomyces lividans* TK24. The amount of the recombinant *Sm*-AA expressed in the *S. lividans* cells was approximately 42-fold higher than that of *Sm*-AA found in the supernatant of *S. mobaraensis*. *Sm*-AA showed high hydrolytic activity towards various *N*-acetyl-L-amino acids and *N*-(middle/long)-chain-fatty-acyl-L-amino acids, with a preference for the acyl derivatives of L-Met, L-Ala, L-Cys, *etc.* with an optimum pH and temperature for reaction of about 7.5 and 50 °C (at pH 7.5).

Key words: aminoacylase; actinomycete; *Streptomyces mobaraensis*; *N*-acyl amino acids

Aminoacylase (*N*-acyl-L-amino-acid amidohydrolase, EC 3.5.1.14) exists in a wide variety of sources, including animal tissues,[1–3] plants,[4] molds,[5,6] and bacteria.[7–20] Most isolated aminoacylases show a substrate specificity towards *N*-acetyl-L-amino acids, including ones isolated from hog kidney,[1] *Aspergillus oryzae*,[5] *Alcaligenes denitrificans* DA181,[7] *Bacillus stearothermophilus*,[10] *Lactococcus lactis* MG1363,[11] and *Streptomyces mobaraensis*, as reported in our previous paper.[14] In practice, aminoacylases, such as from *A. oryzae*, are utilized in the production of L-amino acids from the corresponding acetyl-DL-amino acids.[15,16] On the other hand, some other isolated aminoacylases specifically hydrolyze *N*-long-chain-acyl amino acids, such as those from *Mycobacterium smegmatis*[17,18] and carboxypeptidase G3 from *Pseudomonas* sp.[21] Aminoacylase from *Pseudomonas diminuta* specifically hydrolyzes *N*-long-chain-acyl glutamic acids,[19,20] but the genes of these *N*-long-chain-acyl aminoacylases are yet to be identified. In addition to the short-chain-acyl aminoacylase from *S. mobaraensis*, as above,[14] we recently isolated two other acylases: ε-lysine acylase, with a substrate specificity to various *N*ε-acyl-L-lysines;[22,23] and penicillin V acylase, which efficiently hydrolyzes *N*-lauroyl-L-amino acids, *N*-lauroyl-peptides, and capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) in addition to Penicillin V.[24,25] During the course of the purification of these enzymes from the culture supernatant of *S. mobaraensis*, we isolated another new aminoacylase (hereafter *Sm*-AA), which can efficiently hydrolyze *N*-(middle/long)-chain-acyl-L-amino acids, as well as *N*-acetyl-L-amino acids, with a preference for the acyl derivatives of L-Met, L-Ala, L-Cys, *etc*. An aminoacylase that can hydrolyze both *N*-(middle/long)-chain-fatty-acyl-L-amino acids and *N*-short-chain-fatty-acyl-L-amino acids is yet to be reported, with the exception of carboxypeptidase G3 from *Pseudomonas* sp.[21] Therefore, in this study, we purified and characterized *Sm*-AA. On the basis of the N-terminal amino acid sequence of the purified enzyme, we cloned the *Sm*-AA gene, and sequenced and expressed *Sm*-AA in *S. lividans* TK24 cells for characterization.

Materials and Methods

Materials (chemicals). DEAE Sephadex A-50 and Octyl Sepharose 4 Fast Flow or Octyl Sepharose CL-4B were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Hydroxyapatite (Fast Flow Type) and Hi-Trap Chelating HP (5 ml) were from Wako Pure Chemical Industries (Osaka, Japan) and GE Healthcare Bio-Sciences AB respectively. Various *N*α-acetyl-amino acids were from either Wako or Sigma-Aldrich (St. Louis, MO). *N*-Butyryl-L-Met, *N*-hexanoyl-L-Met, *N*-octanoyl-L-Met, *N*-decanoyl-L-Met, *N*-lauroyl-L-Met, *N*-myristoyl-L-Met, and *N*-palmitoyl-L-Met were prepared by a

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Abbreviations: ACY1, aminoacylase-1 from porcine kidney; BSA, bovine serum albumin; DTT, dithiothreitol; *Fs*-PM, peptidase M20 from *Frankia* sp.; NBRC, National Institute of Technology and Evaluation Biological Resource Center; PCMB, *p*-chloromercuribenzoic acid; SDAP, succinyl-diaminopimelate desuccinylase from *Hemophilus influenzae*; *Sa*-PP, putative peptidase from *S. avermitilis*; *Sm*-AA, aminoacylase from *S. mobaraensis*; *Sp*-HP, hypothetical protein from *Streptomyces pristinaespiralis*

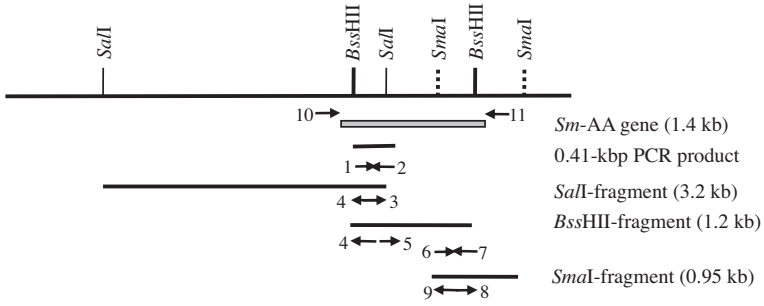

Fig. 1. Scheme for Cloning of the *Sm*-AA Gene.
Arrows 1–12 show the primers used, which correspond to the numbers shown in Table 1.

method reported elsewhere.[26] Briefly, the corresponding acid chloride (Tokyo Chemical Industry, Tokyo) was added drop-wise to 0.6 M L-Met dissolved in 2 N NaOH on ice with vigorous stirring to obtain the product as a precipitate. Then the precipitate was put in contact with *n*-hexane to extract acids, followed by rinsing with pure water, and finally freeze-drying.

We used pSH19[27] as an expression vector, a gift from Dr. M. Kobayashi, University of Tsukuba. Restriction endonucleases and T4 DNA ligase were from either TAKARA BIO (Ohtsu, Japan) or Toyobo (Osaka). An Advantage™-GC genomic PCR Kit (BD Biosciences, San Jose, CA) and KOD Plus DNA Polymerase (Toyobo) were used to amplify fragments including the partial and whole *Sm*-AA sequences respectively. Colony PCR was performed using Go Taq® Green Master Mix (Promega, Madison, WI). Agarose-LE (Classic Type) was from Nacalai Tesque (Kyoto, Japan). A Quantum Prep® Plasmid Miniprep Kit (Bio-Rad, Hercules, CA) was used in the purification of plasmids. All other reagents were of analytical grade and were purchased from either Wako, Sigma-Aldrich, or Nacalai Tesque.

Buffers. The following buffers were used: buffer A, 50 mM Tris–HCl buffer, pH 7.5; buffer B, 25 mM Tris–HCl buffer, pH 7.5; and buffer C, 20 mM sodium phosphate buffer, pH 7.0. Buffer A was mainly used in enzyme assays. Buffer B was an elution buffer used in chromatographic separation of the enzyme and suspension of the cells. Buffer C was used during separation of the enzyme from the cell-free extract on a Hi Trap Chelating HP column.

Enzyme assays. The reaction was carried out at 37 °C using 15 mM *N*-acetyl-L-Met or 3 mM *N*-lauroyl-L-Met as the substrate in buffer A. The L-Met produced was assayed by the ninhydrin method. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of L-Met at 37 °C at pH 7.5 in 1 min. Most of the data obtained were represented as mean values of triplicate experiments. Maximum S.D. values were 7% of the mean values.

Microorganisms, cultivation of wild-type cells, and enzyme purification. S. mobaraensis NBRC (IFO) 13819, a type culture of the NBRC (National Institute of Technology and Evaluation Biological Resource Center, Chiba, Japan) was cultured using a method reported previously.[22] The culture supernatant obtained was used in the purification of the enzyme. All the purification procedures were carried out at 4 °C. First, ammonium sulfate was added to the culture supernatant from *S. mobaraensis* (1,000 ml) to attain 60% saturation in order to precipitate the enzyme. The precipitate, which was recovered by centrifugation at 18,000 × *g* for 20 min, was dissolved in 50 ml of buffer B containing 50 mM NaCl, and was dialyzed against the same buffer. Then the dialyzed solution was loaded on a DEAE Sephadex A-50 column (30 × 2.6 cm i.d.) and eluted by a linear increase in the NaCl concentration in buffer B from 50 to 500 mM at a flow rate of 0.24 ml/min. The active fractions were collected and then placed on an Octyl Sepharose 4 Fast Flow column (34 × 1.6 cm i.d.) for elution by a linear decrease in the NaCl concentration in buffer B from 1 to 0 M, followed by elution with buffer B at a flow rate of 0.27 ml/min. The active fractions were collected, concentrated, and dialyzed against buffer A. The N-terminal amino acid sequence of the purified enzyme was analyzed by the method reported previously using a protein sequencer (Applied Biosystems, Model 491, Foster City, CA).[25]

Table 1. Primers Used in This Study

Number	Sequence
1	5′-TCGACATCTGCCGCGACCTGATCC-3′
2	5′-GTTGACGGTGAAGGAGAACCCGCC-3′
3	5′-ATCCACGGGCACACGGACGTCGTC-3′
4	5′-GGCGAGCTTCTCGGCCACGTACTC-3′
5	5′-CCGCTACCTGGTGGACAAGCACCC-3′
6	5′-TTCCTCGCCGACCTGGACCGGATC-3′
7	5′-CGTCCACTCCGTGGAACATCCCGG-3′
8	5′-CCGGGATGTTCCACGGAGTGGACG-3′
9	5′-GATCCGGTCCAGGTCGGCGAGGAA-3′
10	5′-GACCAGCGGGTTCTTGCGGGAGTG-3′
11	5′-CGACACGGAGGGAATCGGGCTACG-3′
12	5′-AAGCTTAGCAACGGAGGTACGGACATGGGAGT-CATCTTGCGCTTGC-3′
13	5′-TGCGGGTGGTGCGGGTCGCCCAACGATAGGAA-TTC-3′

We purified *Sm*-AA from the cell-free extract using several chromatographic columns. Briefly, first we disrupted the cells suspended in buffer B using Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT). The supernatant of the suspended cells was first subjected to ammonium sulfate (70% saturation) precipitation, and subsequently the enzyme was separated by DEAE Sephadex A-50 chromatography in a way similar to that used in the separation of the enzyme from the culture supernatant, as explained above. The active fraction obtained from the DEAE Sephadex column was loaded on a hydroxyapatite column (30 × 1.6 cm i.d.) and eluted by changing the sodium phosphate buffer (pH 7.5) concentration from 10 to 200 mM. The active fraction eluted at about 50 mM sodium phosphate buffer was further purified by Octyl Sepharose CL-4B chromatography in a way similar to that used above. Finally, the enzyme, dissolved in buffer B containing 1 M NaCl, was loaded on a Hi-Trap Chelating HP column and eluted using 50 mM EDTA in buffer C to obtain the purified enzyme.

Cloning and sequencing of the Sm-*AA gene.* Total genomic DNA was isolated from the *S. mobaraensis* cells by a method reported by Kieser *et al.*[28] The *E. coli* DH5α cells, which were used as a host strain in DNA manipulation, were grown in LB medium, as described previously.[25] The scheme for the cloning of the *Sm*-AA gene is shown in Fig. 1 and the primers used are listed in Table 1. To identify an internal partial sequence of the *Sm*-AA gene, PCR was carried out using genomic DNA as a template with oligonucleotides 1 and 2 as sense and anti-sense primers respectively. Oligonucleotide 1 was designed on part of the revealed N-terminal amino acid sequence, and oligonucleotide 2 was designed on the internal amino acid sequence of a hypothetical protein from *S. avermitilis* (GenBank accession no. BAB69369)—the N-terminal amino acid sequence, which, according to a BLAST search, shows a high homology (70%) to that of *Sm*-AA, as explained below. The amplified PCR product of 0.41 kbp was purified, inserted into the pUC118 *Hin*cII/BAP vector, and used in the transformation of the *E. coli* DH5α cells. The positive clones were isolated and sequenced using an ABI Prism 310 DNA sequencer (Perkin-Elmer, Wellesley, MA).

For hybridization, 15 μg of genomic DNA isolated from *S. mobaraensis* cells was digested using *Mlu*I, *Bss*HII, *Nco*I, *Bam*HI, *Sal*I, *Pst*I, *Sac*I, *Kpn*I, *Sph*I, and *Sma*I, and was transferred to a Hybond-N+ membrane (Amersham Biosciences, Little Chalfont, UK). For the first hybridization, a probe was prepared by PCR reaction with a PCR DIG Labeling Mix (Roche Diagnostics, Basel, Switzerland) using the plasmid containing the sequence of the 0.41-kbp PCR product as a template and oligonucleotides 1 and 2 as the primers.

Hybridization was performed at 55 °C for 4 h with the DIG-labeled probe, and immunological detection was then performed according to the manufacturer's protocol (Roche Diagnostics). The 3.2-kbp *Sal*I-digested DNA fragments, which showed strong signal intensity, were isolated, self-ligated, and used as a template for inverse PCR using oligonucleotides 3 and 4 as the sense and anti-sense primers respectively. The obtained PCR product of 3 kbp was cloned, and its nucleotide sequence, including the initiation codon, was determined by the method described above. Similarly, the 1.2-kbp *Bss*HII-digested DNA fragments, which also showed strong signal intensity, were isolated, self-ligated, and used as a template for inverse PCR using oligonucleotides 5 and 4 as the sense and anti-sense primers respectively, and then the 0.85-kbp PCR product was obtained and sequenced. Two primers, oligonucleotides 6 and 7, were designed by the sequence that exists in the vicinity of the 3′-end of the *Bss*HII fragment. These primers were used in the preparation of a DIG-labeled probe by PCR reaction using the 0.85-kbp PCR product as a template for the second hybridization. The 0.95-kb *Sma*I-digested fragments with a strong signal intensity were separated, self-ligated, and used as a template for inverse PCR using oligonucleotides 8 and 9 as the sense and anti-sense primers respectively and a 0.75-kbp fragment was amplified and sequenced. Finally, to confirm the overall ORF sequence, the 1.4-kbp PCR product, including the full sequence of the *Sm*-AA gene, was amplified using oligonucleotides 10 and 11, which were designed by the DNA sequence of the upstream 5′-end and downstream 3′-end of the *Sm*-AA gene. The PCR product was inserted into pUC118 *Hin*cII/BAP, and the resulting plasmid, pUC118-*Sm*AA, was sequenced.

Nucleotide sequence data analysis and sequence alignment were done using a DNASIS® Pro (Hitachi Software Engineering, Tokyo) program. Sequence similarity to *Sm*-AA was analyzed using NCBI BLAST for protein sequences. The signal peptide sequence was predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

Construction of the expression plasmid. The *Sm*-AA gene was amplified by PCR using pUC118-*Sm*AA as a template and oligonucleotides 12 and 13 as the sense and antisense primers respectively. Oligonucleotide 12 was designed to add the *Hin*dIII site and the ribosomal binding site to the 5′-end of the *Sm*-AA gene. Oligonucleotide 13 was designed to add the *Eco*RI site to the 3′-end of the *Sm*-AA gene. The amplified 1.4-kbp fragment was inserted into pUC118 *Hin*cII/BAP and sequenced. The 1.4-kbp fragment was inserted into the *Hin*dIII and *Eco*RI sites of an expression vector, pSH19.[27] The resulting plasmid, pSH19-*Sm*AA, was introduced into *S. lividans* protoplasts, and the positive clones were selected by a method described by Fukatsu *et al.*[29]

Cultivation of recombinant cells and purification of Sm-*AA.* The transformed *S. lividans* cells were cultivated and expressed by a method reported by Fukatsu *et al.*[29] Recombinant *S. lividans* cells prepared as above were pre-cultured in a 100-ml shaking flask containing 30 ml of TSB medium (Kanto Chemical, Tokyo) that was supplemented with 20 μg/ml of thiostrepton at 30 °C with shaking at 120 strokes/min for 1 d. Then 1 ml of the pre-culture was inoculated into 50 ml of the same culture medium in two 100-ml shaking flasks, followed by cultivation at 30 °C with shaking at 120 strokes/min. Following a 2-d cultivation, isovaleronitrile or ε-caprolactam was added to the culture medium as an inducer at final concentrations of 0.1% (v/v) and 0.1% (w/v) respectively. Then cultivation was allowed to continue for another day, after which the cells were recovered by centrifugation at 5,000 × *g* for 15 min at 4 °C. Then approximately 1 g of wet *S. lividans* cells was washed with 10 mM Tris–HCl buffer (pH 7.5) and suspended in 20 ml of buffer A. The cell suspension was sonicated using an Ultrasonic Disruptor UD-201 (Tomy Seiko, Tokyo), followed by centrifugation at 8,000 × *g* for 20 min at 4 °C. The cell-free extract was used in purification of the recombinant *Sm*-AA. All purification procedures were carried out at 4 °C. The protein precipitate, obtained by adding ammonium sulfate at 50% saturation, was dissolved in 30 ml of buffer B containing 1 M NaCl, and it was then dialyzed against the same solution and subjected to Octyl Sepharose 4 Fast Flow gel chromatography to elute the enzyme in a way similar to that for the purification of wild-type *Sm*-AA.

Characterization of Sm-*AA.* The enzyme properties of *Sm*-AA were studied primarily using purified recombinant *Sm*-AA at a final concentration of 1.5 μg/ml by a method similar to that reported in our previous work.[14] When wild-type *Sm*-AA was used, its final concentration was usually 0.4 μg/ml.

The activity dependency on pH for the wild-type and recombinant *Sm*-AAs was studied at 37 °C using 50 mM acetate buffer (pH 4.0–6.0), 50 mM Tris–HCl buffer (pH 6.0–8.0), and 50 mM borate buffer (pH 8.0–10.0). The optimal reaction temperature was determined by conducting reactions for 10–20 min using purified wild-type and recombinant *Sm*-AAs in buffer A containing 15 mM *N*-acetyl-L-Met as the substrate in a temperature range of 16 to 70 °C. The pH stability of the recombinant enzyme was studied by incubating the recombinant enzyme for 1 h at 37 °C at various pH levels. In order to measure thermal stability, the recombinant enzyme was incubated in buffer A for 1 h in a temperature range of 16–70 °C. To determine the effects of the reagents on stability, the recombinant *Sm*-AA was incubated in buffer A at 37 °C for 15 min containing the following: 1 mM *p*-chloromercuribenzoic acid (PCMB); iodoacetamide; β-mercaptoethanol; dithiothreitol (DTT); GSH; L-Cys; 1, 10-phenanthroline; EDTA; and various 0.5 mM metal ions ($ZnCl_2$, $NiCl_2$, $CoCl_2$, $CaCl_2$, $CuSO_4$, $MgSO_4$, $FeSO_4$, and $MnSO_4$). Then the remaining enzyme activity was assayed at 37 °C using 15 mM *N*-acetyl-L-Met as the substrate. The effects of metal ions on the reactivation of the enzyme were studied as follows: First the recombinant *Sm*-AA was dialyzed against buffer A containing 10 mM of 1, 10-phenanthroline for 6 h at 4 °C to prepare a metal-free enzyme, followed by dialysis against a sufficiently large amount of buffer A for 36 h with five changes of the buffer. Then the resulting metal-free enzyme was pre-incubated with various metal ions ($ZnCl_2$, $NiCl_2$, $CoCl_2$, $CaCl_2$, $CuSO_4$, $MgSO_4$, $FeSO_4$, and $MnSO_4$), usually at a final concentration of 0.005 mM for 1 h at 37 °C, and the enzyme activity was measured by adding a final dose of 15 mM *N*-acetyl-L-Met as the substrate. The metal-free enzyme was also pre-incubated in buffer A containing final concentrations of 0.0005, 0.005, 0.05, 0.5, and 2.5 mM $ZnCl_2$ for 1, 20, and 55 h at 37 or 4 °C before the measurement of enzyme activity.

The substrate specificity of *Sm*-AA was studied using recombinant *Sm*-AA towards various 15 mM *N*-acetyl-amino acids, 3 mM *N*-lauroyl-amino acids, and 3 mM *N*-saturated-acyl-L-Mets with carbon chain lengths of C2, C4, C6, C8, C10, C12, C14, and C16.

The initial reaction kinetics for hydrolysis of *N*-acetyl-L-Met was determined as follows: A 10-μl aliquot of purified recombinant *Sm*-AA solution was added to 0.99 ml of a substrate solution at various final concentrations of 2–20 mM at 37 °C. At appropriate times, a 10-μl portion of the reaction mixture was withdrawn, and the concentration of L-Met formed was assayed as described previously to determine the initial reaction rate.

Analytical methods. Protein concentration was determined using a BCA Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, IL) with bovine serum albumin (BSA) as the standard. SDS–PAGE was performed by the Laemmli method.[30] The protein bands were stained with Quick-CBB Plus (Wako Pure Chemical Industries). Blue Native PAGE was performed using a NativePAGE™ NOVEX® Bis-Tris Gel System (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol.

Results and Discussion

Purification of wild-type Sm-*AA from culture supernatant*

The culture supernatant showed enzyme activity of approximate 8.4 U/ml in terms of *N*-acetyl-L-Met

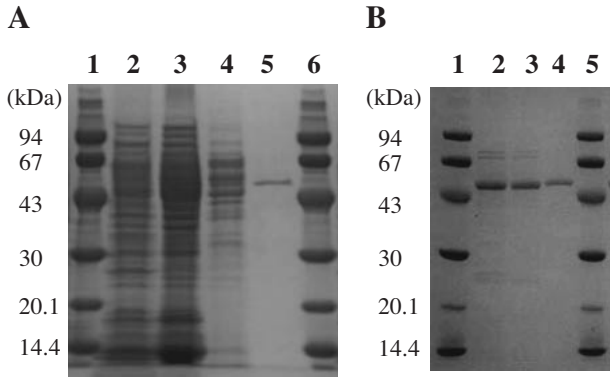

Fig. 2. Results of SDS–PAGE Analysis of the Protein Fractions Obtained at Various Purification Steps of Wild-Type and Recombinant *Sm*-AAs.

A, Wild-type *Sm*-AA. Lanes 1 and 6, marker proteins; lane 2, culture filtrate; lane 3, ammonium sulfate precipitate; lane 4, the active fraction from DEAE Sephadex A-50 column chromatography; lane 5, that from Octyl Sepharose 4 Fast Flow column chromatography. B, Recombinant *Sm*-AA. Lanes 1 and 5, marker proteins; lane 2, cell-free extract; lane 3, ammonium sulfate precipitate; lane 4, the active fraction from Octyl Sepharose 4 Fast Flow column chromatography. As marker proteins, phosphorylase (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.

hydrolyzing activity. On DEAE Sephadex A-50 column chromatography, the active fraction of wild-type *Sm*-AA appeared at a NaCl concentration of 480 mM in buffer B; Octyl Sepharose 4 Fast Flow column chromatography, the enzyme was eluted at an NaCl concentration of 200 mM in buffer B. According to the SDS–PAGE results for the active fraction collected from the final column, as shown in Fig. 2A, the enzyme was homogeneous, with an apparent molecular mass of approximately 50 kDa. Blue Native PAGE analysis showed a single band with an estimated molecular weight of approximately 55 kDa (data not shown). These findings indicate that the enzyme was monomeric. Table 2 lists a purification summary. The enzyme was purified approximately 500-fold, with a yield of 10%. The specific activity of the purified enzyme was 440 U/mg, with 15 mM *N*-acetyl-L-Met dissolved in buffer A at 37 °C as the substrate. The purified enzyme also showed hydrolytic activity towards 3 mM *N*-lauroyl-L-Met, with a specific activity of 65 U/mg, under the same conditions. Aminoacylases that exhibit high hydrolytic activity towards both *N*-acetyl-L-Met and *N*-lauroyl-L-Met have not yet been reported, as far as we know, and this motivated us to identify its gene and characteristics of the enzyme in detail. The first 15 N-terminal amino acid residues of the purified enzyme were determined to be GAENEVVDICRDLIR, and then were used in cloning the gene.

Purification of wild-type Sm-*AA from the cell-free extract*

Sm-AA purified from the cell-free extract was homogeneous on the SDS–PAGE gel (data not shown). The N-terminal amino acid residue of *Sm*-AA extracted from the SDS–PAGE gel was GAENEVVDICRDLIR, the same as the first 15 residues of the enzyme purified from the culture supernatant.

Table 2. Purification Summary of Wild-Type Aminoacylase from *S. mobaraensis*

Purification step	Protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification (fold)
Culture supernatant	9418	8429	100	0.89	1.0
Ammonium sulfate precipitation	1037	4953	59	4.8	5.4
DEAE Sephadex A-50	94.8	2507	30	27	30
Octyl Sepharose 4 Fast Flow	1.8	806	9.7	440	494

Cloning of the Sm-*AA gene*

The complete ORF of *Sm*-AA contained a DNA sequence of 1,383-bp encoding 460 amino acids (data not shown). The ORF started with an ATG codon and terminated at the TGA codon. The nucleotide sequence of the *Sm*-AA gene was submitted to the DDBJ database (accession no. AB478961). The deduced amino acid sequence is shown in Fig. 3. In Fig. 3, the N-terminal amino acid sequence determined from *Sm*-AA purified from the culture supernatant and cell-free extract is located in the deduced amino acid sequence and is double-underlined. As Fig. 3 shows, the N-terminal peptide of *Sm*-AAs purified from the supernatant and cell-free extract was truncated at the 29th position. Signal P 3.0 analysis suggested that *Sm*-AA is not a secretory protein, due to lack of the signal peptide. These findings suggest that *Sm*-AA is an intracellular enzyme. The reason that *Sm*-AA was secreted in the supernatant of *S. mobaraensis* is not known at present. One possible reason is that *Sm*-AA, an intracellular enzyme, was leaked during cultivation in a way similar to *S. mobaraensis* ε-lysine acylase, as reported in our previous paper.[22,23]

The whole amino acid sequence of *Sm*-AA was used to search for protein sequence homology by the BLAST program using a Genbank database. The *Sm*-AA gene sequence showed 89, 88, 67, 29, and 25% identity with those for a hypothetical protein from *Streptomyces pristinaespiralis* (*Sp*-CHP, Genbank accession no. EDY63492), a putative peptidase from *S. avermitilis* (*Sa*-PP, Genbank accession no. BAB69369), peptidase M20 from *Frankia* sp. (*Fs*-PM, Genbank accession no. ABD12016), succinyl-diaminopimelate desuccinylase from *H. influenzae* (SDAP, Genbank accession no. P44514), and aminoacylase-1 from porcine kidney (ACY1, Genbank accession no. P37111), all of which belong to the peptidase M20 family. In Fig. 3, the alignment of the deduced amino acid sequence of *Sm*-AA with those of the enzymes belonging to the peptidase M20 family mentioned above is shown. *Sm*-AA possesses fully conserved residues of metal-binding sites, H107, D139, E174, E201, and H435, and catalytic sites, D109 and E173 (shaded in Fig. 3), for the peptidase M20 family, which requires Zn^{2+} for the expression of enzyme activity. In addition, the three conserved amino acid motifs in the peptidase M20 family were also found: (i) [L, F, V]-x-[S, G, A]-H-x-D-x-V, and (ii) [G, D]-x-x-[Y, W, F]-[G, A]-x-G-x-x-D; and, (iii) x-E-E (boxed in Fig. 3).

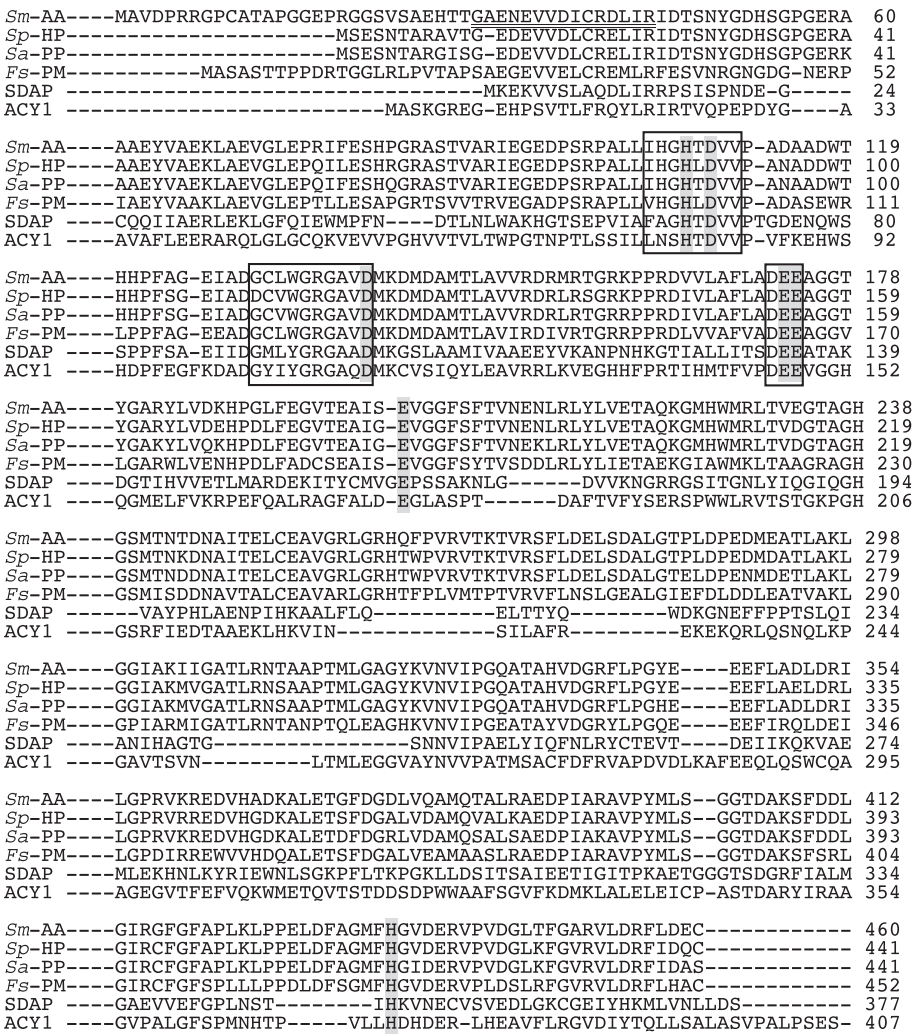

Fig. 3. Comparison of the Amino Acid Sequence of *Sm*-AA with Those of Enzymes Belonging to the Peptidase M20 Family.

The enzymes shown are *Sm*-AA, isolated in this study, a hypothetical protein from *Streptomyces pristinaespiralis* (*Sp*-HP, Genbank accession no. EDY63492), a putative peptidase from *S. avermitilis* (*Sa*-PP, Genbank accession no. BAB69369), peptidase M20 from *Frankia* sp. (*Fs*-PM, Genbank accession no. ABD12016), succinyl-diaminopimelate desuccinylase from *H. influenzae* (SDAP, Genbank accession no. P44514), and aminoacylase-1 from porcine kidney (ACY1, Genbank accession no. P37111). The N-terminal amino acid sequence of the purified *Sm*-AA is double-underlined.

Expression of recombinant Sm-*AA and purification*

S. lividans cells harboring pSH19-*Sm*AA were cultured in TSB medium containing 20 μg/ml of thiostrepton, and *Sm*-AA was expressed by the addition of either 0.1% (v/v) isovaleronitrile or 0.1% (w/v) ε-caprolactam, as described in "Materials and Methods." No *Sm*-AA activity was detected in the culture supernatant, in contrast to that from *S. mobaraensis*, as mentioned previously, and the whole enzyme was recovered from the cell-free extract. The amounts of the recombinant *Sm*-AA obtained following induction with ε-caprolactam and isovaleronitrile were 33 U/ml-culture and 136 U/ml-culture respectively. The *Sm*-AA activity obtained with isovaleronitrile was approximately 42-fold higher than that of the wild-type *Sm*-AA recovered from the culture supernatant of *S. mobaraensis* 3.2 U/ml-culture. It should be noted here that the culture supernatant contained some other enzymes in addition to *Sm*-AA that might have hydrolyzed *N*-acetyl-L-Met, such as aminoacylase, reported in our previous paper.[14] Therefore, the amount of wild-type *Sm*-AA in the culture supernatant discussed above was evaluated from the *N*-lauroyl-L-Met hydrolyzing activity of the culture supernatant (0.47 U/ml) with the specific activities measured for the purified enzyme towards *N*-acetyl-L-Met (440 U/mg) and *N*-lauroyl-L-Met (65 U/mg). No activity was detected when wild-type *S. lividans* cells or those harboring pSH19 were used. The cell-free extract was first subjected to Octyl Sepharose 4 Fast Flow column chromatography, in which the active enzyme fraction was eluted near a NaCl concentration of 0 M. Although the SDS–PAGE result appeared to indicate that the *Sm*-AA fraction recovered from the Octyl Sepharose 4 Fast Flow column was homogeneous, as shown in Fig. 2B, detailed protein sequence analysis later revealed that it was actually composed of a mixture of two proteins of *Sm*-AA—N-terminal amino acid residue A11 and V23—and was different from that of the wild-type *Sm*-AA, G30. The reason for the difference in the length of the N-terminal peptides truncated between purified wild-type and recombinant *Sm*-AAs is not known at present and remains, as a future project. We subjected the mixture of two recombinant *Sm*-AAs to column chromatography using Macro-Prep Ceramic

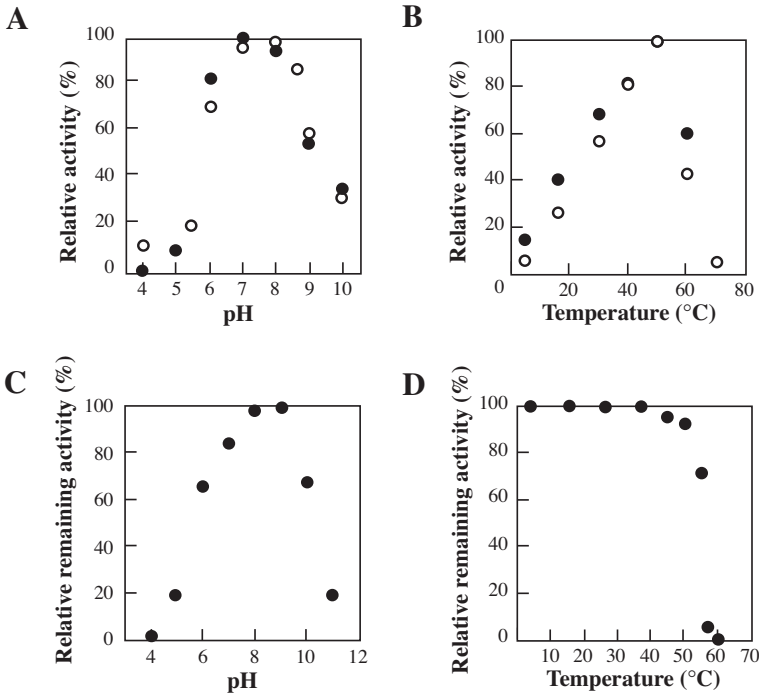

Fig. 4. Some Properties of *Sm*-AA.

A, pH Dependency of hydrolysis activity towards *N*-acetyl-L-Met at 37 °C for recombinant *Sm*-AA (closed keys) and wild-type enzyme (open keys). B, Optimal reaction temperature for hydrolysis of 15 mM *N*-acetyl-L-Met at pH 7.5 using recombinant *Sm*-AA (closed keys) and wild-type enzyme (open keys) incubated at 37 °C for 1 h. C, pH Dependency of stability for *Sm*-AA. D, Thermal stability of the enzyme at pH 7.5 using recombinant *Sm*-AA.

Table 3. Purification Summary of Recombinant Aminoacylase from *S. lividans* TK24

Purification step	Protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification (fold)
Cell-free extract	142	13600	100	96	1.0
Ammonium sulfate precipitation	55	7088	52	129	1.3
Octyl Sepharose 4 Fast Flow	4.6	2438	18	530	5.5

hydroxyapatite Type II (Bio-Rad), then eluted it, and this linearly increased the phosphate buffer concentration from 5 to 200 mM. The two *Sm*-AA enzymes, however, eluted at the same 40 mM phosphate buffer concentration and could not be separated. In Table 3, the purification summary of the recombinant *Sm*-AA from *S. lividans* is presented. The specific activities of the purified recombinant *Sm*-AA towards substrates of 15 mM *N*-acetyl-L-Met and 3 mM *N*-lauroyl-L-Met were 530 and 72 U/mg respectively, and were slightly higher than those for the purified wild-type enzyme, as mentioned previously, 440 and 65 U/mg. The difference in the length of the N-terminal peptides truncated above might have caused the difference in specific activities between the purified wild-type and recombinant *Sm*-AAs.

Some properties of recombinant Sm-*AA*

Figure 4A–D shows the pH dependency of activity, optimal reaction temperature, pH stability, and thermal stability of the recombinant enzyme, of which the results from the wild-type *Sm*-AA are also shown in Fig. 4A and B for comparison. The optimal pH for the reaction was in a range of 7 to 8 at 37 °C for both recombinant and wild-type *Sm*-AAs (Fig. 4A), although the tendency was slightly different between the two *Sm*-AAs in the acidic pH region. The optimal reaction temperature was approximately 50 °C (Fig. 4B) for both *Sm*-AAs. Recombinant *Sm*-AA was stable in a pH range of 8 to 9 at 37 °C (Fig. 4C) at temperatures below 40–45 °C at pH 7.5 (Fig. 4D).

As shown in Table 4, the remaining activity for the enzyme treated by 1, 10-phenanthroline was approximately 10% as compared to that for the native enzyme, suggesting that *Sm*-AA is a metalloenzyme. EDTA, which is known to show weaker affinity for Zn^{2+} than 1, 10-phenanthroline, did not affect enzyme stability. The effects of metal ions on the stability of the enzyme were studied at a final concentration of 0.5 mM. Metal ions such as Ca^{2+} and Mg^{2+} did not affect the remaining activity, while Fe^{2+} decreased it by 90%. The remaining activity of the enzyme was slightly decreased by the addition of Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Mn^{2+}, and strongly by Fe^{2+} (data not shown).

The effects of metal ions on reactivation of the metal-free enzyme were studied under various pre-incubation conditions. Table 5 shows the effects of pre-incubation for 1 h at 37 °C in the presence of various metal ions at a final concentration of 0.005 mM on recovery of the activity of metal-free recombinant *Sm*-AA. Metal ions such as Zn^{2+} and Ni^{2+} reactivated the enzyme to some extent, taking the remaining activity of the enzyme incubated in buffer A as control (100%). With increasing the final metal ion concentrations to 0.5 mM, the remaining activity was slightly decreased by pre-incubation with Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+},

Table 4. Effects of Various Reagents on the Enzyme Activity of Recombinant *Sm*-AA

Reagents[a]	Relative activity (%)
Control[b]	100
PCMB	108
Iodoacetamide	85
β-Mercaptoethanol	81
DTT	105
GSH	106
L-Cys	95
1, 10-Phenanthroline	10
EDTA	106

[a]The concentration of the reagents at the first incubation was 1 mM.
[b]The activity of the enzyme in the absence of reagents was taken to be 100%.

Table 5. Effects of Various Metal Ions on Recovery of the Activity of Metal-Free Recombinant *Sm*-AA

Metal ions[b]	Relative remaining activity (%)
Control	100[a]
$ZnCl_2$	120
$NiCl_2$	110
$CoCl_2$	82
$CaCl_2$	86
$CuSO_4$	93
$MgSO_4$	96
$FeSO_4$	86
$MnSO_4$	88

[a]The remaining activity for the metal-free enzyme pre-incubated in buffer A without the addition of metal ions, at 37 °C for 1 h was taken as the control (100%).
[b]The metal-free enzyme was pre-incubated in buffer A, containing a final concentration of 0.005 mM metal ions at 37 °C for 1 h before the measurement of enzyme activity.

and strongly with Fe^{2+} (data not shown) as reported by some investigators,[13,31] while with decreasing metal ion concentrations, the remaining activity of the metal-free enzyme was increased, in particular by pre-incubation with Zn^{2+}. Table 6 shows the effects of the Zn^{2+} concentration on the reactivation of *Sm*-AA. The relative remaining activity was decreased to 39% by incubation with 2.5 mM Zn^{2+}, while the enzyme was re-activated markedly after 20-h and 55-h pre-incubation at 4 °C in the presence of 0.0005 mM Zn^{2+}, indicating that Zn^{2+} is a requisite metal ion for the expression of enzyme activity. The enzyme activity was not fully restored by the addition of Zn^{2+}, probably because the removal process of Zn^{2+} by 1, 10-phenanthroline includes an irreversible process[32,33] although the details are not known at present.

Table 7 summarizes the substrate specificity towards various *N*-acetyl-amino acids and *N*-lauroyl-amino acids using recombinant *Sm*-AA. Here, the substrate concentrations for *N*-acetyl-amino acids were 15 mM, while those for *N*-lauroyl-amino acids were 3 mM, taking into consideration the low solubility. The enzyme efficiently catalyzed *N*-acetyl-amino acids with a preference for the derivatives of L-Met, L-Cys, L-Ala, *etc.*, as well as *N*-lauroyl-amino acids, with a preference for the derivatives of L-Ala, L-Met, L-Ser, and Gly. The specific activities towards *N*-lauroyl-Gly and *N*-lauroyl-L-Lys were higher than those towards *N*-acetyl-Gly and *N*-acetyl-L-Lys. Furthermore, as shown in Table 8, *Sm*-AA

Table 6. Effects of Zn^{2+} Ion on Recovery of the Activity for Metal-Free Recombinant *Sm*-AA

Metal ions	Relative remaining activity (%)
Control	100[a]
2.5 mM $ZnCl_2$	39[b]
0.5 mM $ZnCl_2$	55[b]
0.05 mM $ZnCl_2$	84[b]
0.005 mM $ZnCl_2$	115[b]
0.0005 mM $ZnCl_2$	120[b]
0.0005 mM $ZnCl_2$	136[c]
0.0005 mM $ZnCl_2$	157[d]

[a]The activity of the metal-free enzyme pre-incubated in buffer A without the addition of metal ions under the same conditions in the corresponding experiment in the presence of $ZnCl_2$ was taken as the control (100%).
[b,c,d]The metal-free enzyme was pre-incubated in buffer A, containing different final $ZnCl_2$ concentrations, at 37 °C for 1 h ([b]), 20 h ([c]), and 55 h ([d]) before measurement of enzyme activity.

Table 7. Substrate Specificity of Recombinant *Sm*-AA towards Various *N*-Acetyl- and *N*-Lauroyl-amino Acids

Amino acid	Specific activity (U[a]/mg)	
	N-Acetyl-amino acid[b]	*N*-Lauroyl-amino acid[c]
L-Arg	66	4
L-His	59	6
L-Lys	0	14
L-Asp	8	—
L-Glu	0	—
L-Ala	194	96
L-Asn	31	6
L-Cys	412	17
L-Gln	14	—
Gly	8	34
L-Ile	—	0
L-Leu	60	0
L-Met	530 (173[c])	72
L-Phe	19	0
L-Pro	0	0
L-Ser	—	36
L-Trp	0	0
L-Tyr	0	0
L-Val	24	0

[a]One unit was defined as the amount of enzyme required to hydrolyze 1 µmol of substrate in buffer A in 1 min at 37 °C.
[b]Substrate concentration, 15 mM.
[c]Substrate concentration, 3 mM.

Table 8. Substrate Specificity of Recombinant *Sm*-AA towards Various *N*-Acyl-L-methionines

Substrate[a]	Specific activity (U[b]/mg)
N-Acetyl-L-Met	173
N-Butyryl-L-Met	169
N-Hexanoyl-L-Met	175
N-Octanoyl-L-Met	251
N-Decanoyl-L-Met	89
N-Lauroyl-L-Met	72
N-Myristoyl-L-Met	106
N-Parmitoyl-L-Met	105

[a]Substrate concentration, 3 mM.
[b]One unit was defined as the amount of enzyme required to hydrolyze 1 µmol of the substrate in buffer A in 1 min at 37 °C.

efficiently hydrolyzed 3 mM *N*-acyl-L-Met, which has a carbon chain length of C2 to C16. The specific activity for hydrolysis towards 3 mM *N*-acetyl-L-Met, shown in Table 8, was approximately 1/3, that towards 15 mM *N*-acetyl-L-Met, shown in Table 7. The kinetics of

the hydrolysis of *N*-acetyl-L-Met was studied to make clear the difference in specific activity at different substrate concentrations. The substrate dependency of the initial reaction rate followed Michaelis-Menten kinetics (data not shown), and the *K*m value for hydrolysis of *N*-acetyl-L-Met was determined to be 11.3 ± 1.3 mM with V_{max} of 750 ± 51 μmol/(mg · min) from the Lineweaver-Burk plot. The ratio of the initial rate for hydrolysis of 3 mM to that of 15 mM *N*-acetyl-L-Met was evaluated to be 1/2.7 using the kinetic values determined, similar to the experimental results above.

Sm-AA was characterized by its high reactivity towards *N*-(middle/long)-chain-fatty-acyl-L-amino acids as well as *N*-acetyl-L-amino acids. To date, several long-chain aminoacylases that catalyze *N*-(middle/long)-chain-acyl-L-amino acids have been isolated and characterized. Among them, long-chain-acyl aminoacylase from *M. smegmatis* shows the ability to hydrolyze *N*-decanoyl-, lauroyl-, myristoyl-, palmitoyl-, stearoyl-, and arachidoyl-aspartic acid; but it does not hydrolyze *N*-acetyl-, butyl-, hexanoyl-, or octanoyl-aspartic acid.[17,18] Short-chain-acyl aminoacylase from *M. smegmatis* hydrolyzes *N*-acetyl-aspartic acid but hydrolytic activities are extremely low towards *N*-butyl-, hexanoyl-, octanoyl-, decanoyl-, and palmitoyl-aspartic acid.[17,18] *N*-Long-chain-acyl aminoacylase I from *P. diminuta* hydrolyzes only *N*-(middle/long)-chain-fatty-acyl-glutamic acids.[19] *N*-Long-chain-acyl aminoacylase II from *P. diminuta* hydrolyzes *N*-(middle/long)-chain-fatty-acyl-amino acids.[20] These *N*-long-chain-acyl aminoacylases show no activity towards *N*-acetyl-amino acids.[19,20] Carboxypeptidase G3 from *Pseudomonas* sp. acts only on *N*-fatty-acyl-DL-glutamic acids with a chain length of C2 to C16, and it does not hydrolyze *N*-octanoyl-DL-amino acids for the octanoyl derivatives of amino acids, with the exception of DL-glutamic acid.[21] The genes for the enzymes that catalyze *N*-(middle/long)-chain-acyl-L-amino acids are yet be identified with the noted exception of penicillin V acylase from *S. mobaraensis*, which has been analyzed by our group, and has been found to hydrolyze not only penicillin V but also *N*-lauroyl amino acids, although it does not act on *N*-acetyl-L-amino acids.[24,25] On the other hand, a number of short-chain-acyl aminoacylases reportedly hydrolyze *N*-acetyl-L-amino acids, such as those from hog kidney,[1] *A. oryzae*,[5] *A. denitrificans* DA181,[7] *B. stearothermophilus*,[10] *L. lactis* MG1363,[11] and *S. mobaraensis*.[14] The genes for some of the aminoacylases have been analyzed,[10,11,34–36] but the 3D conformations have not, with the exception of porcine kidney aminoacylase.[36]

Hence, it would be interesting to compare the 3D structures of short-chain-acyl aminoacylases, long-chain-acyl aminoacylases, and *Sm*-AA, and this is a future task.

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