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# Discovery and application of a new enzyme *N*-acylamino acid racemase

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**Abstract**

A novel enzyme, *N*-acylamino acid racemase (NAAR) which catalyzes the interconversion of the enantiomers of *N*-acylamino acid, but does not act on amino acids, has been found in the actinomycetes *Streptomyces atratus* Y-53 and *Amycolatopsis* sp. TS-1-60, isolated from soil. These strains also produced L- and D-aminoacylases simultaneously. Furthermore, another 13 strains of actinomycetes with NAAR activity were observed from the type culture collection of the Institute for Fermentation, Osaka (IFO).

Thermostable *N*-acylamino acid racemase from *Amycolatopsis* sp. TS-1-60, a rare actinomycete strain selected for its ability to grow on agar plates incubated at 40°C, was purified to homogeneity and characterized. The enzyme was stable at 55°C for 30 min and catalyzed the racemization of optically active *N*-acylamino acids such as *N*-acetyl D- or L-methionine, *N*-acetyl-L-valine, *N*-acetyl-L-tyrosine and *N*-chloroacetyl-L-valine. In addition, this enzyme also catalyzed the racemization of the dipeptide L-alanyl-L-methionine. The optically active amino acids, *N*-alkyl-amino acids and ethyl ester derivatives of *N*-acetyl-D and L-methionine, however, were not racemized. Enzyme activity was markedly enhanced by the addition of divalent metal ions such as $Co^{2+}$, $Mn^{2+}$ and $Fe^{2+}$ and was inhibited by the addition of EDTA and PCMB.

The NAAR gene from *Amycolatopsis* sp. TS-1-60, consists of an open reading frame of 1104 nucleotides, which specifies a 368-amino acid protein with a molecular weight of 39,411. No significant sequence homology was found between the DNA sequence or the deduced amino acid sequence of NAAR and those of known racemases and epimerases in data bases. However, comparison of the amino acid sequences of mandelate racemase and NAAR showed that NAAR has partial homology with the catalytic and metal ion binding sites of that enzyme. The amount of NAAR produced by an *E. coli* transformant hosting a T7 expression plasmid was 1100-fold more than that produced by *Amycolatopsis* sp. TS-1-60.

Bioreactors for the production of optically active amino acids were constructed with DEAE Toyopearl-immobilized NAAR and D- or L-aminoacylase. D- or L-Methionine was continuously produced with a high yield from *N*-acetyl DL-methionine by these bioreactors. 

*Keywords:* *N*-Acylamino acid racemase; D-Amino acid; Chiral resolution; D-Aminoacylase

## 1. Introduction

Almost all L-amino acids have been industrially produced by the fermentation methods using a high-breeding strain. On the other hand, some of them have been produced by enzymatic methods. For example, L-methionine is continuously produced from *N*-acetyl DL-methionine by immobilized L-minoacylase [1] In this method, only *N*-acetyl-L-methionine is stereospecifically deacetylated by L-aminoacylase and converted into L-methionine. After separating

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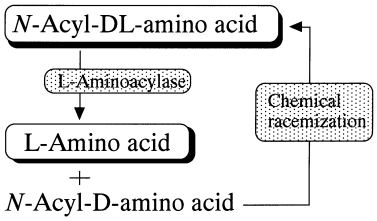


Fig. 1. Production of L-amino acid from *N*-acyl-DL-amino acid by L-aminoacylase.

L-methionine from the reaction mixture, the remaining *N*-acetyl-D-methionine is racemized by chemical methods under severe conditions [2–5] to be used as the starting materials for the next cycle of the process (Fig. 1). The yield of optically resolved amino acids is less than 50% because the isomer which is not deacylated in the starting material remains in the reaction mixture. If *N*-acylamino acid could be selectively racemized by an enzyme in the presence of an optically active amino acid under mild conditions, *N*-acyl-DL-amino acid will be totally converted into L-amino acid by the racemase in cooperation with L-aminoacylase without any separation procedure. In this system, D-amino acid will be produced from the same starting material with a high yield by using D-aminoacylse instead of L-aminoacylase (Fig. 2). This method using the racemase in combination with D- or L-aminoacylase will increase the yield of optical resolution of amino acids from less than 50 to 100%.

A number of amino acid racemases [6–12] have been found in micro-oganisms and intensively studied. However, there have been no reports of a racemase which converts optically active *N*-acylamino acids into the corresponding enantiomers but does not act on optically active amino acids. Therefore, we tried to screen for the enzyme activity in micro-organisms isolated from soil and type culture collections of the Institute for Fermentation, Osaka (IFO).

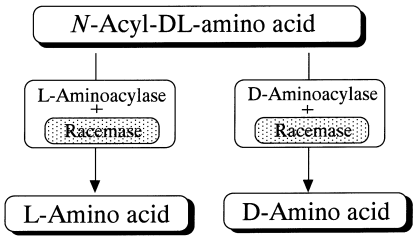


Fig. 2. Highly effective production of optically active amino acid from *N*-acyl-DL-amino acid by D- or L-aminoacylase and *N*-acylamino acid racemase.

This review describes a strategy for screening, characterization, gene cloning, and application of a new enzyme *N*-acylamino acid racemase (NAAR).

## 2. Screening of NAAR

### *2.1. Screening of micro-organisms producing NAAR*

The strategy for screening for NAAR is shown in Fig. 3 [13]. If a micro-organism produces NAAR in a culture broth containing *N*-acetyl-D-methionine as a substrate, a part of the substrate will be converted into the L-isomer, *N*-acetyl-L-methionine. The *N*-acetyl L-methionine produced must be converted into L-methionine by L-aminoacylase which specifically deacetylates *N*-acetyl-L-amino acids. However, it is believed that there are three possible pathways for the production of L-methionine from *N*-acetyl D-methionine: (1) *N*-acetyl D-methionine is converted into D-methionine by D-aminoacylase, and D-methionine is converted into L-methionine by amino acid racemase; (2) D-methionine is converted into the corresponding 2-keto acid by D-amino acid oxidase or D-amino acid aminotransferase, and then the 2-keto acid converted into L-methionine by amino acid aminotransferase; and (3) micro-organisms themselves are L-methionine producers. For evaluating these possibilities, we selected micro-organisms that produce L-methionine from *N*-acetyl-D-methionine, but not from D-methionine.

### *2.2. Distribution of micro-organisms producing NAAR*

About 49,000 strains of bacteria, actinomycetes, yeasts, and fungi were screened through the detection of enzyme activity. We discovered NAAR activity in actinomycetes (Table 1). Some bacterial strains produced a small amount of L-methionine from *N*-acetyl-D-methionine. However, NAAR was not detected from these strains; they had D-aminoacylase and amino acid racemase activities. The results suggested that D-methionine was probably produced from

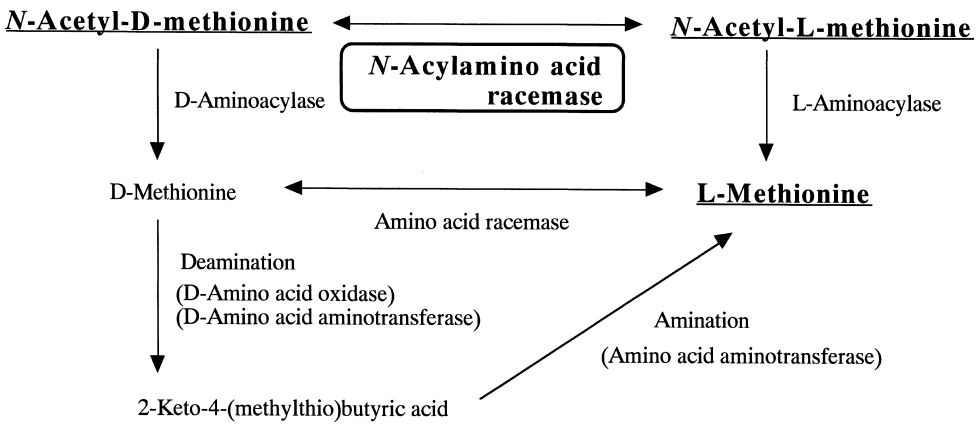


Fig. 3. Possible pathways for production of L-methionine from *N*-acetyl-D-methionine.

*N*-acyl-D-methionine by D-aminoacylase and converted into L-methionine by amino acid racemase. We also found that some fungi produced L-methionine and the corresponding 2-keto acid (2-keto-4-(methylthio) butyric acid). D-Methionine was assumed to be produced by D-aminoacylase and converted into the α-keto acid by D-amino acid oxidase or D-amino acid aminotransferase. The keto acid was converted into L-methionine by amino acid aminotransferase. Type cultures of actinomycetes from the IFO were screened and 13 strains were found to show the activity of NAAR. Among these strains shown in Table 2, *Streptomyces atratus* Y-53 and Amycolatopsis sp. TS-1-60, which were isolated from a soil sample, produce the enzyme at high levels.

*Streptomyces atratus* Y-53 and *Amycolatopsis* sp. TS-1-60 grown in Trypticase Soy Broth without supplementation produced NAAR. However, the addition of 0.1% *N*-acetyl-DL-methionine to the medium resulted in a two fold increase in NAAR production. All strains that showed enzyme activity, produced D- and L-aminoacylases simultaneously.

There have been many reports of amino acid racemases [14]. Yorifugi et al. [9] reported that the arginine racemase of *Pseudomonas graveolens* racemized ε-*N*-acetyllysine and δ-*N*-acetylornithine but not α-*N*-acetylated derivatives of lysine and ornithine; the α-amino group of the substrate was required to be free for the enzymatic racemization. Inagaki et al. [11] reported that ε-*N*-acetyllysine and methionine and

Table 1
Distribution of micro-organisms producing *N*-acyl amino acid racemase

| Organism | Source | Number tested | Number of producer |
|---|---|---|---|
| Bacteria | IFO | 686 | 0 |
| | Soil | 4800 | 0 |
| Actinomycetes | IFO | 850 | 13 |
| | Soil | 42000 | 2 |
| Fungi | IFO | 1067 | 0 |
| Yeasts | IFO | 785 | 0 |

Table 2
*N*-Acylamino acid racemase producers

| Micro-orgarism | L-Methionine produced (mM) |
|---|---|
| *Streptomyces atratus* Y-53 | 12.7 |
| *Amycolatopsis* sp. TS-1-60 | 17.2 |
| *Actinomadura roseoviolacea* IFO 14098 | 1.2 |
| *Actinomyces aureomonopodiales* IFO 13020 | 1.6 |
| *Jensenia canicruria* IFO 13914 | 2.1 |
| *Amycolatopsis orientalis* IFO 12806 | 1.4 |
| *Sebekia benihana* IFO 14309 | 3.9 |
| *Streptomyces coelescens* IFO 13378 | 4.2 |
| *Streptomyces celluloflavus* IFO 13780 | 3.2 |
| *Streptomyces alboflavus* IFO 13196 | 2.9 |
| *Streptomyces aureocirculatus* IFO 13018 | 2.3 |
| *Streptomyces diastatochromogenes* IFO 13389 | 1.8 |
| *Streptomyces spectabilis* IFO 13424 | 2.8 |
| *Streptomyces tuirus* IFO 13418 | 3.8 |
| *Streptomyces riseoaurantiacus* IFO 13381 | 2.7 |

some other amino acids were good substrates for an amino acid racemase purified from *Aeromonas caviae* with broad substrate specificity. We have found NAAR activity in some actinomycetes strains belonging to the genus *Streptomyces*, *Actinomadura*, *Actinomyces*, *Jensenia*, *Amycolatopsis*, and *Sebekia*. There is great interest in the distribution of micro-organisms with enzyme activity and the role of the racemization in the micro-organisms.

## 3. Purification and properties of thermostable NAAR from *Amycolatopsis* sp. TS-1-60

### 3.1. Purification of NAAR

Thermostable NAAR from *Amycolatopsis* sp. TS-1-60 [15], a rare actinomycete strain selected for its ability to grow on agar plates incubated at 40°C, was purified to homogeneity (Table 3). The enzyme, purified about 338-fold, was obtained with a yield of 27.8% from the cell extract. The cell extract contained 1950U of *N*-acylamino acid racemase, 11,000U of L-aminoacylase and 23,300U of D-aminoacylase. Heat treatment inactivated 60–70% of the initial activity of both aminoacylases and denatured about 70% of the total protein. L-Aminoacylase and D-aminoacylase were removed by Butyl-Toyopearl and DEAE-Toyopearl column chromatography, respectively. The purified enzyme gave a single peak on gel filtration and a single band on SDS-PAGE.

### 3.2. Substrate specificity

The purified enzyme catalyzed the racemization of various *N*-acylamino acids but did not act on the corresponding amino acids (Table 4 ). *N*-Propionyl-D- and L-methionine, *N*-chloroacetyl-L-valine, *N*-butyryl-L-methionine and *N*-acetyl-L-methionine were effective substrates. *N*-Acyl-derivatives of aromatic amino acids such as *N*-acety-L-tyrosine, *N*-acetyl-D-phenylalanine and *N*-chloroacetyl-D-phenylalanine also served as good substrates. In addition, the enzyme also catalyzed the racemization of the L-methionine moiety of dipeptide, L-alanyl-L-methionine. Among *N*-acylated derivatives of methionine and valine, NAAR racemized L-isomer better than D-isomer except *N*-formylated methionine. On the other hand, NAAR acted on the D-isomer of *N*-acetylated derivatives of alanine, leucine and phenylalanine better than the corresponding L-isomer. These results indicate that the amino acid moieties of *N*-acylamino acids affect on stereoselectivity of NAAR.

To examine the relationship between enzyme activity and the carbon chain length of acyl residues in *N*-acylated amino acids, *N*-formyl-, *N*-acetyl-, *N*-propionyl- and *N*-butylyl-methionine were used as substrates. As shown in Table 4, *N*-propionyl-methionine was the best substrate for activity followed by *N*-acetyl-, *N*-butylyl- and *N*-formyl-methionine. The enzyme did not catalyze the racemization of methyl or ethyl esters of *N*-acetyl-methionine or *N*-methyl derivatives of phenylalanine and leucine. These results indicate that NAAR requires an *N*-acyl residue and free carboxylic acid linked to a chiral-carbon in the substrate for enzyme activity. *N*-Acetyl derivatives of glutamic acid, glutamine, aspartic acid, asparagine, lysine, histidine and proline were not racemized. The apparent $K_m$ values calculated from Hanes–Woolf plots were 18.5 mM for *N*-acetyl-L-methionine and 11.3 mM for *N*-acetyl-D-methionine. The $V_{max}$ values for the racemization of *N*-acetyl-L-methionine and

Table 3
Purification of *N*-acylamino acid racemase from *Amycolatopsis* sp. TS-1-60[a]

| Step | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) |
|---|---|---|---|---|
| Crudeextract | 134000 | 1950 | 0.015 | 100 |
| Heat treatment | 39900 | 1550 | 0.039 | 79.5 |
| Butyl-Toyopearl | 537 | 1140 | 2.12 | 58.5 |
| DEAE-Toyopearl | 194 | 781 | 4.03 | 40.1 |
| G3000SW | 107 | 542 | 5.07 | 27.8 |

[a] Assay mixture: 25 mM *N*-acetyl-methionine, 50 mM Tris–HCl buffer (pH 7.5), 2 mM $CoCl_2$ and 2 units L-aminoacylase in a total volume of 500 μl. The assay mixture was incubated at 30°C for 5 min.

Table 4
Substrate specificity of *N*-acylamino acid racemase from *Amycolatopsis* sp. TS-1-60[a]

| Substrate | Relative activity (%)[b] | Substrate | Relative activity (%) |
|---|---|---|---|
| D-Met | 0 | L-Met | 0 |
| Fr-D-Met | 20 | Fr-L-met | 20 |
| Ac-D-Met | 100 | Ac-L-Met | 150 |
| Pr-D-Met | 138 | Pr-L-Met | 196 |
| Bu-D-Met | 85 | Bu-L-Met | 112 |
| Ac-D-Ala | 28 | Ac-L-Ala | 5 |
| Bz-D-Ala | 5 | Bz-L-Ala | – |
| Ac-D-Leu | 23 | Ac-L-Leu | 14 |
| CA-D-Leu | – | CA-L-Leu | 41 |
| Ac-D-Phe | 69 | Ac-L-Phe | 28 |
| CA-D-Phe | 53 | CA-L-Phe | 14 |
| Ac-D-Trp | 14 | Ac-L-Trp | 14 |
| Ac-D-Tyr | – | Ac-L-Tyr | 92 |
| Ac-D-Val | 27 | Ac-L-Val | 83 |
| CA-D-Val | 63 | CA-L-Val | 179 |
| *N*-Carbamoyl-D-Met | 2 | L-Ala-L-Met | 12 |
| Ac-D-Met methylester | 0 | Ac-L-Met methylester | 0 |
| *N*-Methyl-D-Phe | 0 | *N*-Methyl-L-Phe | 0 |
| *N*-Methyl-D-Ieu | – | *N*-Methyl-L-Leu | 0 |
| D-Val | 0 | L-Val | 0 |

[a] Abbreviation: Ac: *N*-Acetyl; Fr: *N*-Formyl; Pr: *N*-Propionyl; Bu: *N*-Butyryl; Bz: *N*-Benzoyl; CA: *N*-Chloroacetyl; –: not determined. Assay mixture: 25 mM substrate, 50 mM Tris–HCl buffer (pH 7.5), 2 mM $CoCl_2$ and 50 ml enzyme solution in a total volume of 500 ml. The reaction mixture was incubated at 30°C for 5 min.

[b] Activity for Ac-D-Met was taken as 100.

*N*-acetyl-D-methionine were 1.51 and 0.98 mM/mm, respectively.

### 3.3. Effect of metal ions and inhibitors

NAAR was significantly activated by the addition of $Co^{2+}$ and $Mn^{2+}$ while the addition of $Pb^{2+}$, $Al^{3+}$ and $Cu^{2+}$ noticeably inhibited activity (Table 5). Enzyme activity was strongly inhibited by the addition of sulfhydryl reagents such as *p*-chloromercuribenzoic acid (PCMB). The addition of hydroxylamine and L-penicillamine, which are known as inhibitors of pyridoxal 5′-phosphate dependent enzymes, did not affect enzyme activity. The addition of an excess of EDTA, a metal-chelating reagent, strongly inhibited enzyme activity, suggesting that this racemase is a metalloenzyme.

### 3.4. Physicochemical properties

Table 6 shows the properties of the NAAR from *Amycolatopsis* sp. TS-1-60 and that from *Streptomyces*

Table 5
Effect of metal ions on the actitivity of *N*-acylamino acid racemase from *Amycolatopsis* sp. TS-1-60[a]

| Metal | Concentration (mM) | Relative activity (%) |
|---|---|---|
| None | | 100 |
| $MgSO_4 \cdot 7H_2O$ | 1 | 139 |
| $MnSO_4 \cdot 6H_20$ | 1 | 386 |
| $FeSO_4 \cdot 7H_2O$ | 1 | 104 |
| | 10 | 382 |
| $CoCl_2 \cdot 6H_20$ | 1 | 496 |
| $NiSO_4 \cdot 6H_20$ | 1 | 157 |
| $CuSO_4 \cdot 5H_2O$ | 1 | 150 |
| | 10 | 0 |
| $ZnSO_4 \cdot 7H_2O$ | 1 | 154 |
| $Al_2(SO_4)_3 \cdot 16H_20$ | 1 | 71 |
| | 10 | 0 |
| $PbCl_2$ | 1 | 7 |
| $SnSO_4$ | 1 | 71 |
| $BaCl_2 \cdot 2H_20$ | 1 | 75 |
| $CaCl_2 \cdot 2H_2O$ | 1 | 25 |
| NaCl | 1 | 104 |
| KCl | 1 | 82 |

[a] The reaction was carried out under standard assay conditions except for the substitution of various metal salts for $CoCl_2$.

Table 6
Comparison of *N*-acylamino acid racemase from *Amycolatopsis* sp. TS-1-60 with that from *Streptomyces* atratus Y-53

| | *Amycolatopsis* sp. TS-1-60 | *Streptomyces atratus* Y-53 |
|---|---|---|
| Molecular weight: native enzyme subunit | 300 K | 244 K |
| | 40 K | 41 K |
| Isoelectric point | 4.2 | 4.8 |
| Optimamum pH | 7.5 | 6.5–8.0 |
| Optimum temperature | 50°C | 40°C |
| Thermostability (30 min) | 55°C | 40°C |
| Inhibitor | PCMB, EDTA | PCMB, EDTA, *N*-Ethylmaleimide |
| Stimulator | $Co^{2+}$, $Mn^{2+}$, $Fe^{2+}$ | $Co^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Zn^{2+}$ |

*atratus* Y-53 [16]. The thermostability of the racemase from *Amycolatopsis* sp. TS-1-60 was greater than that of the racemase from *Streptomyces atratus* Y-53 (Fig. 4). The $M_r$ values of the native enzyme and the subunit from *Amycolatopsis* sp. TS-1-60 were estimated to be 300,000 by gel filtration and 40,000 by SDS-PAGE, respectively. These results suggest that the enzyme is composed of eight identical subunits. On the other hand, the enzyme from *Streptomyces atratas* Y-53 is considered to be composed of six identical subunits. Both the subunits seem to be composed of a very similar protein judging from the $M_r$ values and the analyses of the $NH_2$-terminal amino acid sequences in which 20 of 24 amino acid residues are identical.

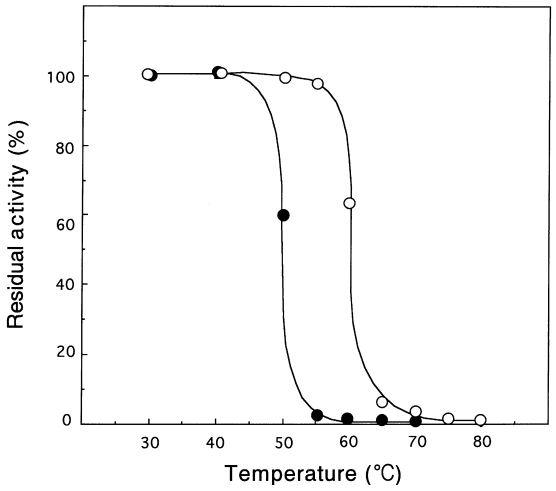


Fig. 4. Effect of temperature on stability of *N*-acylamino acid racemase NAAR in 50 mM Tris–HCl buffer (pH 7.5), was incubated for 30 min at the temperature indicated. The remaining activity was measured under standard assay conditions. Symbols: ○, *Amycolatopsis* sp. TS-1-60; ●, *Streptomyces atratus* Y-53.

Amino acid racemases are generally stimulated by the addition of PLP or FDA, while some enzymes such as glutamate racemase [17,18], aspartate racemase [19,20], proline racemase [21] and diaminopimelate epimerase [22] do not need a cofactor for activity. Mandelate racemase [23], on the other hand, requires metal cations such as $Mg^{2+}$, $Co^{2+}$ and $Ni^{2+}$ for activity, and NAAR also requires $Co^{2+}$, $Mn^{2+}$ and $Fe^{2+}$. It is possible that the mechanism of racemization catalyzed by NAAR resembles that of mandelate racemase, due to the metal-ion requirement for enzyme activity.

### *3.5. Change in optical rotation*

Fig. 5 shows changes in the optical rotation of *N*-acetyl-D- and L-methionine incubated in a reaction mixture with the racemase for 2 h. Equal amounts of L- and D-methionine from the each incubation mixture were detected by HPLC in each reaction mixture.

## 4. Cloning and expression of the NAAR gene

### *4.1. Cloning of the NAAR gene*

The amino acid sequences of peptide fragments P1 and P2 obtained from digestion of NAAR by lysyl-endpeptidase were determined to be as follows:

P1; Val–Trp–Ile–Gly–Ser

P2; Gly–Ala–Val–Gln–Ile–Val–Asn–Ile–Lys–Pro–Gly–Arg–Val–Gly–Gly–Tyr–Leu–Glu–Ala–Arg–Arg–Val–His–Asp–Val–Cys–Ala–Ala–His–Gly–Ile–Pro

The following oligonucleotide deduced from the underlined portion of peptide fragment P2 were synthe-

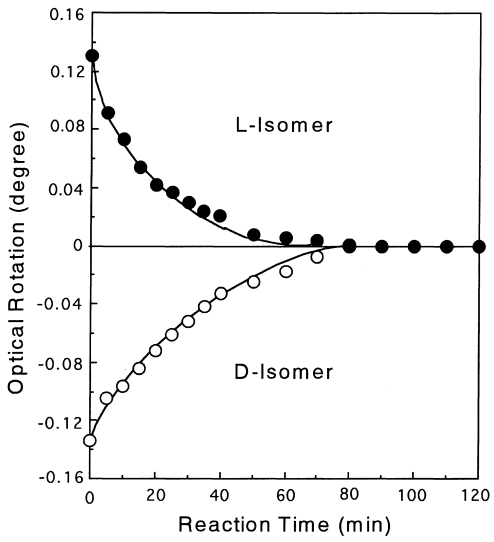


Fig. 5. Changes in optical rotation of *N*-Acetyl-D or L-Methionine during incubation with *N*-acylamino acid racemase from *Amycolatopsis* sp. TS-1-60. Symbols: ○, D-isomer; ●, L-isomer.

sized and used as a probe for Southern hybridization. 5′-CAGATCGTRAACATCAAGCC-3′ (R: G or C).

A clone having the NAAR gene was screened for with an antibody probe against NAAR. Thirteen positive plaques were obtained from among approximately 200,000 plaques. The inserted DNA fragments of two recombinant phages, λ-8 and λ-9, were hybridized with the oligonucleotide probe deduced from the amino acid sequence of the peptide fragment P2. The restriction maps of λ-8 and λ-9 are shown in Fig. 6. An oligonucleotide sequence corresponding to the amino acid sequence of peptide fragment P2 was found in these inserted DNAs, but sequence corresponding to the $NH_2$-terminal portion of NAAR was not found. For cloning of the complete NAAR gene, about 40,000 plaques from the DNA library were screened by plaque hybridization using the *Eco*RI–*Kpn*I fragment (0.56 kb), which is a part of the inserted DNA in λ-9, as a probe. After screening, four positive plaques were obtained. The recombinant phage λ-44 had the 1.2 kb *Eco*RI fragment which hybridized with the probe.

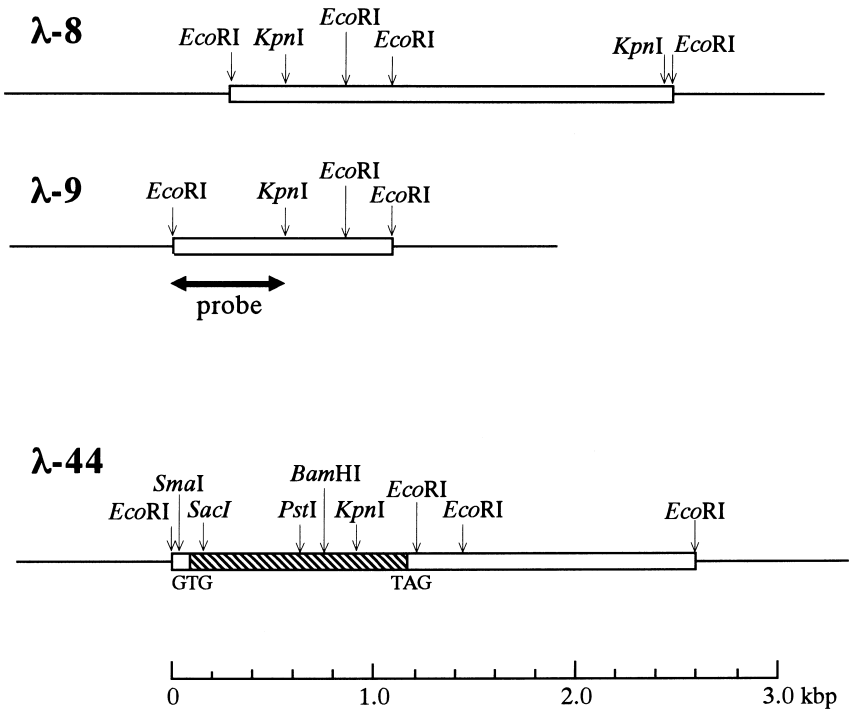


Fig. 6. Restriction maps of the inserted fragments in recombinant phages λ-8, λ-9 and λ-44. Open box: the DNA fragment from *Amycolatopsis* sp. TS-l-60 genomic DNA. Shadowed box: the *N*-acylamino acid racemase gene. ↔: the DNA fragment used as a probe for Southern hybridization.

### *4.2. DNA sequence of the NAAR gene*

Various deletion mutants of the 1.2 kb insert were generated and sequenced [24]. We found an open reading frame (ORF) encoding 368 amino acids which contained the same $NH_2$-terminal amino acid sequence, except for the first methionine, as that of NAAR from *Amycolatopsis* sp. TS-1-60. The amino acid sequences of peptide fragments P1 and P2 were found in the C-terminal and the internal amino acid sequences deduced from the DNA sequence, respectively (underlined in Fig. 7). The ORF started with an unusual initiation codon, GTG, and the overall average of the guanine plus cytosine content of the coding region was 69.7%. The molecular weight of the encoded protein (39,411) determined from the deduced

```
        10         20         30         40         50         60         70         80         90
AATTCCCCGGGTGACCGGCTTCGACCGAGCCGGCTTTTACGTGATCTCCAAGGAGGAGCAGTGAAACTCAGCGGTGTGGAACTGCGCCGG
                                                   SD       MetLysLeuSerGlyValGluLeuArgArg
       100        110        120        130        140        150        160        170        180
GTGCAGATGCCGCTCGTCGCCCCGTTCCGGACTTCGTTCGGCACCCAGTCGGTCCGCGAGCTCTTGCTGCTGCGCGCGGTCACGCCGGCC
ValGlnMetProLeuValAlaProPheArgThrSerPheGlyThrGlnSerValArgGluLeuLeuLeuArgAlaValThrProAla
       190        200        210        220        230        240        250        260        270
GGCGAGGGCTGGGGCGAATGCGTGACGATGGCCGGTCCGCTGTACTCGTCGGAGTACAACGACGGCGCGGAACACGTGCTGCGGCACTAC
GlyGluGlyTrpGlyGluCysValThrMetAlaGlyProLeuTyrSerSerGluTyrAsnAspGlyAlaGluHisValLeuArgHisTyr
       280        290        300        310        320        330        340        350        360
TTGATCCCGGCGCTGCTGGCCGCGGAAGACATCACCGCGGCGAAGGTGACGCCGCTGCTGGCCAAGTTCAAGGGCCACCGGATGGCCAAG
LeuIleProAlaLeuLeuAlaAlaGluAspIleThrAlaAlaLysValThrProLeuLeuAlaLysPheLysGlyHisArgMetAlaLys
       370        380        390        400        410        420        430        440        450
GGCGCGCTGGAGATGGCCGTGCTCGACGCCGAACTCCGCGCGCACGAGAGGTCGTTCGCCGCCGAACTCGGATCGGTGCGCGATTCTGTG
GlyAlaLeuGluMetAlaValLeuAspAlaGluLeuArgAlaHisGluArgSerPheAlaAlaGluLeuGlySerValArgAspSerVal
       460        470        480        490        500        510        520        530        540
CCGTGCGGCGTTTCGGTCGGGATCATGGACACCATCCCGCAACTGCTCGACGTCGTGGGCGGATACCTCGACGAGGGTTACGTGCGGATC
ProCysGlyValSerValGlyIleMetAspThrIleProGlnLeuLeuAspValValGlyGlyTyrLeuAspGluGlyTyrValArgIle
       550        560        570        580        590        600        610        620        630
AAGCTGAAGATCGAACCCGGCTGGGACGTCGAGCCGGTGCGCGCGGTCCGCGAGCGCTTCGGCGACGACGTGCTGCTGCAGGTCGACGCG
LysLeuLysIleGluProGlyTrpAspValGluProValArgAlaValArgGluArgPheGlyAspAspValLeuLeuGlnValAspAla
       640        650        660        670        680        690        700        710        720
AACACCGCCTACACCCTCGGCGACGCGCCGCAGCTGGCCCGGCTCGACCCGTTCGGCCTGCTGCTGATCGAGCAGCCGCTGGAAGAGGAG
AsnThrAlaTyrThrLeuGlyAspAlaProGlnLeuAlaArgLeuAspProPheGlyLeuLeuLeuIleGluGlnProLeuGluGluGlu
       730        740        750        760        770        780        790        800        810
GACGTGCTCGGCCACGCCGAACTGGCCCGCCGGATCCAGACACCGATCTGCCTCGACGAGTCGATCGTGTCGGCGCGCGCGGCGGCGGAC
AspValLeuGlyHisAlaGluLeuAlaArgArgIleGlnThrProIleCysLeuAspGluSerIleValSerAlaArgAlaAlaAlaAsp
       820        830        840        850        860        870        880        890        900
GCCATCAAGCTGGGCGCGGTCCAAATCGTGAACATCAAACCGGGCCGCGTCGGCGGGTACCTGGAAGCGCGGCGGGTGCACGACGTGTGC
AlaIleLysLeuGlyAlaValGlnIleValAsnIleLysProGlyArgValGlyGlyTyrLeuGluAlaArgArgValHisAspValCys
       910        920        930        940        950        960        970        980        990
GCGGCGCACGGGATCCCGGTGTGGTGCGGCGGGATGATCGAGACCGGCCTCGGCCGGGCGGCGAACGTCGCGCTGGCCTCGCTGCCGAAC
AlaAlaHisGlyIleProValTrpCysGlyGlyMetIleGluThrGlyLeuGlyArgAlaAlaAsnValAlaLeuAlaSerLeuProAsn
      1000       1010       1020       1030       1040       1050       1060       1070       1080
TTCACCCTGCCCGGCGACACCTCGGCGTCGGACCGGTTCTACAAAACCGACATCACCGAGCCGTTCGTGCTCTCCGGCGGCCACCTCCCG
PheThrLeuProGlyAspThrSerAlaSerAspArgPheTyrLysThrAspIleThrGluProPheValLeuSerGlyGlyHisLeuPro
      1090       1100       1110       1120       1130       1140       1150       1160       1170
GTGCCGACCGGACCGGGCCTCGGCGTGGCGCCGATTCCGGAGCTGCTGGACGAGGTGACCACGGCAAAGGTGTGGATCGGTTCGTAGCCC
ValProThrGlyProGlyLeuGlyValAlaProIleProGluLeuLeuAspGluValThrThrAlaLysValTrpIleGlySer
      1180       1190       1200       1210       1220       1230       1240       1250       1260
GCTACGAATTCCGGAGGTAGATTTGGTCGGATCGGACCAGCCGGTCCGCACGAGGCCGGATCTACCTTCGGGGGGTGCTGACACCGGTGC
      1270       1280       1290       1300       1310       1320       1330       1340       1350
CGAGCAAACCGCACACGAGTCTGGGACGCGTCCTCGAAGCTCTCGGGGACGTGCTCCTCGAGCCGGTCGCCGTCGGCGCGACACGCGGCG
      1360       1370       1380       1390       1400
GCAGCTCGGCGGGGTGGTGATTCACGACCCGCACGACGACGCGGAATTC
```

Fig. 7. Nucleotide and deduced amino acid sequences of the *N*-acylamino acid racemase gene from *Amycolatopsis* sp. TS-1-60. The underlined amino acid sequences were determined from the *N*-acylamino acid racemase purified from *Amycolatopsis* sp. TS-1-60. SD indicates a possible ribosome-binding sequence. This nucleotide sequence has been submitted to the GSDE, DDBJ, EMBL, and NCBI nucleotide sequence databases under accesstion number D30738.

amino acid sequence was in good agreement with that of NAAR from *Amycolatopsis* sp. TS-1-60 (40,000) is determined by SDS-PAGE. The amino acid composition of NAAR [15] is also in good agreement with that deduced from the sequence data. Consequently, we concluded that this ORF must code the NAAR gene. The upstream sequence reveals the presence of a putative ribosome binding site, AGGAGG, appropriately located in front of the initiation codon. The DNA sequence encoding NAAR was compared with data bases. Although the amino acid sequence of NAAR was compared with those of alanine racemase [25–27], aspartate racemase [28], glutamate racemase [29] and hydantoin racemase [30], no significant similarities were observed. NAAR is a unique racemase that requires divalent metal ions for enzyme activity but not pyridoxal-5′-phosphate. Mandelate racemase from *Pseudomonas putida* is also activated by divalent metal ions [31]. Both enzymes, NAAR and mandelate racemase, consist of eight identical subunits, the molecular weight of which has been determined to be 39,411 and 38,570 [32] from the DNA sequence, respectively. The sequence of NAAR was 9 amino acids longer than that of mandelate racemase which comprises 359 amino acids. Comparison of the amino acid sequences of the two enzymes showed that NAAR has partial homology with the catalytic and metal ion binding sites of mandelate racemace (Fig. 8). Mandelate racemase has an active site containing two distinct general acid/base catalysts, Lys 166 and His 297, and three metal ion binding sites, Asp 195, Glu221 and Glu 247 [33]. We found amino acid sequences similar to those containing the catalytic or metal ion binding sites in mandelate racemase, except for the sequence around His 297 of mandelate racemase, in NAAR (Fig. 8, underlined). Therefore, Lys 163 may

```
MR    1   MSEVLITGLR TRAVNVPLAY PVHTAVGTVG TAPLVLI-DL A-TSAG---- V-V-GHSYLF
NAAR  1   MKLSGV-ELR -R-VQMPLVA PFRTSFGTQS VRELLLLRAV TPAGEGWGEC VTMAGPLYSS

      53  AYTPVALKSL KQLLDDMAAM IVNEPLAPVS LEAMLAKRFC LAGYTGLIRM AAAGIDMAAW
      58  EYNDGAEHVL RHYL--IPAL LAAEDITAAK VTPLLAK-F- -KGH----RM AKGALEMAVL

                                                                    166
      113 DALGKVHETP LVKLLGANAR P-VQ-AYDSH SLDGVKLATE RAVTA-AELG F-RAVKTKIG
      109 DAELRAHERS FAAELGS-VR DSVPCGVSVG IMDTIPQLLD -VVGGYLDEG YVR-IKLKIE
                                                                    163
                                                 195                221
      169 YPALDQDLAV VRSIRQAVGD DFGIMVDYNQ SLDVPAAIKR SQALQQEGVT WIEEPTLQHD
      166 -PGWDVE-PV -RAVRERFGD DVLLQVDANT AYTLGDAPQL AR-LDPFGLL LIEQPLEEED
                                 189                               214
                            247
      229 YEGHQRIQSK LNVPVQMGEN WLGPEEMFKA LSIGACRLAM PDAMKIGG-V TGWIRASALA
      222 VLGHAELARR IQTPICLDES IVSARAAADA IKLGAVQIVN IKPGRVGGYL EA-RRVHDVC
                            240
                  297
      288 QQFGIPM-SS HLFQ-EIS-A HLLAATPTAH W-L--ERL-- D-L-AGSVIE PTLTFEGGNA
      281 AAHGIPVWCG GMIETGLGRA ANVALASLPN FTLPGDTSAS DRFYKTDITE P-FVLSGGHL

      338 VIPDLPGVGI I-WRE--KEI --GK-YL-VU
      340 PVPTGPGLGV APIPELLDEV TTAKVWIGSU
```

Fig. 8. Comparison of amino acid sequence among mandelate racemase and *N*-acylamino acid racemase. The asterisks represent matched amino acid residues between mandelate racemase from *Pseudomonas putida* and *N*-acylamino acid racemase from *Amicolatopsis* sp.TS-l-60.

Table 7
Purification of *N*-acyl amino acid racemase from *E. coli* transformant containing pET3cN[a]

| Step | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) |
|---|---|---|---|---|
| Crude extract | 29820 | 117 | 3.92 | 100 |
| Heat treatment | 15222 | 100 | 6.58 | 86 |
| Butyl-Toyopearl | 3230 | 75.7 | 23.4 | 65 |

[a] Assay mixture: 25 mM *N*-acetyl-methionine, 50 mM Tris–HCl buffer (pH 7.5), 2 mM $COCl_2$ and 2 units L-aminoacylase in a total volume of 500 μl. The assay mixture was incubated at 30°C for 5 min.

be a part of catalytic sites in NAAR and Asp 189, Glu 214 and Glu 247 may be metal ion binding sites in NAAR, respectively.

### *4.3. Overproduction of NAAR*

In order to overexpress the NAAR gene in *E. coli*, the translation initiation codon of the gene was introduced into the *Nde*I site in the translation initiation codon of the T7 $\phi$ gene fragment within the T7 expression plasmid pET3c [34]. An *Nde*I–*Bgl*II fragment containing the NAAR gene was inserted into plasmid pET3c between the *Nde*I site and *Bam*HI site, and thus expression the plasmid pET3cN for the NAAR gene was constructed.

An *E. coli* MM294 transformant containing pET3cN was cultured in a 50 l fermentor for the overproduction of NAAR. The enzyme productivity was 22,300 units/l culture broth which is about 1100-fold higher than that with *Amycolatopsis* sp. TS-1-60. NAAR in soluble form accounted for about 17% of the soluble protein in the *E. coli* transformant.

Table 7 summarizes the purification of NAAR from 296 g (wet cells) of *E. coli* transformant containing pET3cN. The purified NAAR exhibited approximately a sixfold increase in specific activity with a 65% yield from the crude extract of the transformant after two steps: heat treatment and Butyl-Toyopearl column chromatography. The heat treatment denatured about 50% of the soluble protein in the crude extract. The enzyme eluted from the Butyl-Toyopearl column was homogeneous by SDS-PAGE. Although NAAR was purified to homogeneity from the DNA donor strain, *Amycolatopsis* sp. TS-1-60, in four steps, the cloned NAAR could be purified from the transformant by a two-step procedure due to the high-level expression of the NAAR gene. This simple procedure for purification of the cloned enzyme from the transformant is advantageous for industrial purposes. The $NH_2$-terminal amino acid sequence and the amino acid composition of the cloned NAAR were in good agreement with those of the NAAR from the original strain.

## 5. Continuous production of optically active methionine by a bioreactor

### *5.1. Preparation of bioreactor*

NAAR and L-aminoacylase from *Streptomyces atratus* Y-53 or D-aminoacylase from *Amycolatopsis* sp. TS-1-60 were immobilized with DEAE-Toyopearl 650M in 50 mM Tris–HCl buffer (pH 7.5) containing 2 mM $CoCl_2$ [34] (Fig. 9). The DEAE-Toyopearl adsorbed to the enzymes was packed into a column (13 mm × 76 mm) with an outer jacket for maintaining the desired temperature.

### *5.2. Continuous production of optically active methionine*

Fig. 10 shows the time course of optically active methionine production from *N*-acetyl-DL-methionine using bioreactors in which NAAR and D- or L-aminoacylase were immobilized. In the reactor with L-aminoacylase, L-methionine was continuously produced with a yield of more than 99% from *N*-acetyl-DL-methionine for more than 25 h, and no D-methionine was detected in the effluent by HPLC (Fig. 10A). Although a very small amount of *N*-acetyl-DL-methionine was present in the effluent, it was easily separated from L-methionine by recrystallization. The half-life of the bioreactor was 30 days at 30°C (data not shown). In the case of L-methionine production using only L-aminoacylase, the yield of optical

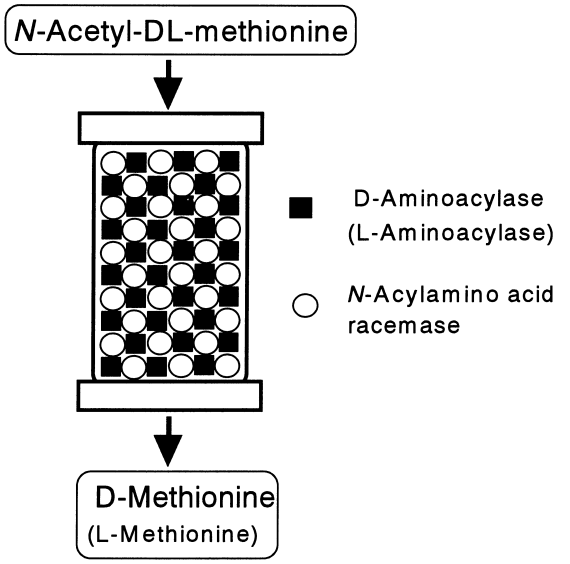


Fig. 9. Bioreactor for the production of optically active amino acids. *N*-Acylamino acid racemase and L- aminoacylase from *Streptomyces atratus* Y-53 [16] or D-aminoacylase from *Amycolatopsis* sp. TS-1-60 [15] were immobilized with DEAE-Toyopearl 650M (Tosoh) in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM $COCl_2$.

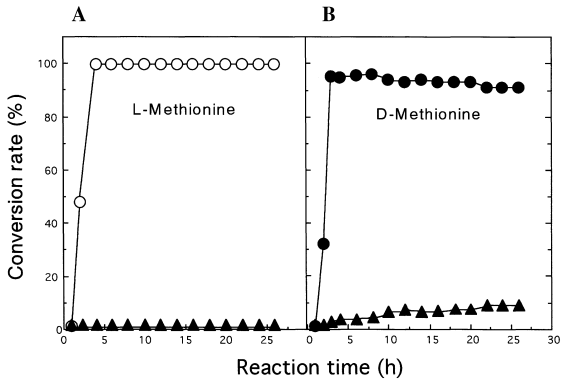


Fig. 10. Continuous production of optically active methionine from *N*-acetyl-DL-methionine by a bioreactor. A solution of 25 mM *N*-acetyl-DL-methionine in 50 mM Tris–HCl buffer (pH 7.5) containing 2 mM $COCl_2$ was applied to a column (13 mm × 76 mm) packed with 10 ml of DEAE-Toyopearl adsorbed with *N*-acylamino acid racemase (30 U) and L-aminoacylase (30 U, A) or D-aminoacylase (30 U, B) at the flow rate of 10 ml/h (sv = 1.0) at 30°C. Symbols: ○, L-Methionine; ●, D-Methionine; ▲, *N* Acetyl-DL-methionine.

resolution is less than 50% because of the stereospecificity of L-aminoacylase.

For D-amino acid production, D-aminoacylase was immobilized on DEAE-Toyopaerl, instead of L-aminoacylase, with NAAR. The yield of D-methionine from *N*-acetyl-DL-methionine was more than 90% and some amont of *N*-acetyl-DL-methionine remained in the reaction mixture (Fig. 10B). However, L-methionine was not detected in the effluent by HPLC. It seemed that the yield of D-methionine production was lower than that of L-methionine production because D-aminoacylase from *Amycolatopsis* sp. TS-1-60 was not as stable as L-aminoacylase from *Streptomyces atratus* Y-53 at 30°C.

## 6. Discussion

Most L-amino acids are produced by fermentation methods using high breeding strains, but D-amino acids are rarely produced by fermentation, with a few exceptions. D-Alanine fermentation by *Brevibacterium lactofermentum* was reported [35]. A D-cycloserine resistant mutant of this strain secreted D-alanine stereoselectively. On the other hand, there have been many enzymatic methods for the production of D-amino acids. Hydantoinase hydrolyzed the D-isomers of various 5-substituted hydantoins to produce the corresponding *N*-carbamyl-D-amino acids which were converted into D-amino acids by decarbamylation [36–44]. Asymmetric hydrolysis of DL-amino acid amides produced D-amino acids by amino acid amido hydrolase [45–48]. Also, stereoselective hydrolysis of *N*-acyl-DL-amino acids with D-aminoacylase was reported for the production of D-amino acids [49–55]. The theoretical yield of D-amino acids from racemates is less than 50% whether the starting material are synthetic compounds or fermentation products. The bioreactor system with a new enzyme, NAAR in combination with D-aminoacylase has been a great advantage in converting whole racemic substrate to D-amino acid with high yield. It is possible in this way to obtain 100% yield of D-amino acid from racemate.

Therefore, a bioreactor using appropriate NAAR and D-aminoacylase appears to be applicable to effective production of D-amino acids from the corresponding *N*-acylamino acids.

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