Discovery of amino acid amides as new substrates for α-amino-ε-caprolactam racemase from Achromobacter obae

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Abstract

Amino acid amide racemizing activity was discovered in α-amino-ε-caprolactam (ACL) racemase (EC 5.1.1.15) from Achromobacter obae. The nucleotide sequence of 1305 bp ACL racemase gene was designed for expression in Escherichia coli and synthesized in a reaction with 46 oligonucleotides by assembly PCR technique. The gene was expressed under the control of the lac promoter, and ACL racemase was purified from E. coli JM109/pACL60 harboring the gene for the enzyme. The enzyme catalyzed the racemization of newly discovered substrates such as 2-aminobutyric acid amide, alanine amide, threonine amide, norvaline amide, norleucine amide, leucine amide, methionine amide, serine amide, and phenylalanine amide. The relative activity towards l-2-aminobutyric acid amide was 2.7% that for l-ACL (350 U/mg), followed by l-alanine amide by 2.1%. Kinetic parameters for the two d- and l-alanine-isomers of amino acid amides (2-aminobutyric acid amide and alanine amide) and l-ACL were obtained from Lineweaver–Burk plots. The Kcat values for l-ACL, d- and l-2-aminobutyric acid amide, and d- and l-alanine amide were calculated to be 360, 40, 13, 9.8 and 7.2 S−1, respectively. The Km values for l-ACL, d- and l-2-aminobutyric acid amide, and d- and l-alanine amide were shown to be 10.1, 3.5, 1.1, 3.4 and 2.5 mM, respectively. The l-enantiomer of 2-aminobutyric acid amide (22.5 mol) was completely racemized by ACL racemase (136 g) in 100 min, and the same amount of l-alanine amide in 120 min. The catalytic efficiency of the newly discovered racemization reaction of 2-aminobutyric acid amide was calculated to be fairly high at about 33% that for l-ACL, while that for alanine amide was about 8.1%. Here, we report that amino acid amides act as new substrates for the ACL racemase.

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Keywords: Amino acid amide; α-ε-Amino-ε-caprolactam Racemase; Assembly PCR

1. Introduction

A number of studies have been made on amino acid racemases acting on amino acid derivatives [1], these include alanine racemase (EC 5.1.1.1) from Salmonella typhimurium [2], glutamate racemase (EC 5.1.1.3) from Pedococcus pentosaceus [3], threonine racemase (EC 5.1.1.6) from Pseudomonas putida [4], arginine racemase (EC 5.1.1.9) from Pseudomonas gravelenta [5], amino acid racemase (EC 5.1.1.10) from P. putida [6] and Aeromonas punctata [7], and aspartate racemase (EC 5.1.1.13) from Streptococcus faecalis [8]. α-Amino-ε-caprolactam (ACL) racemase (EC 5.1.1.15) from Achromobacter obae catalyzes racemization of d- and l-ACL [9,10], and the enzyme was used in combination with l-ACL hydrolase from Cryptococcus laurentii for the industrial l-lysine production from dl-ACL in Japan [11]. Soda et al. reported the enzymatic properties of this ACL racemase and found that the substrates for the enzyme are only ACL, α-amino-β-valerolactam, and α-amino-3-thio-ε-caprolactam; neither α-H-amino acids nor α-H-amino acid amides appeared to be substrates. They even reported that the enzyme does not act on tryptophan amide and leucine amide [12,13].

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α-Amino acids and their derivatives are important commercial products with applications in food supplements, medicines, cosmetics, and as building blocks for compounds with biological or physiological activities [14]. Most of the proteinogenic l-amino acids are efficiently manufactured by fermentation, but α-amino acids are hardly produced by fermentation, with the exception of d-alanine [15].

We previously reported that newly discovered α-stereospecific hydrolases [14,16–19] can be used for the preparation of α-amino acids by an asymmetric hydrolysis of racemic amino acid amides, which are prepared by hydration of the corresponding amino nitriles synthesized from an aldehyde and cyanide by the Strecker synthesis [19,20]. The amino acids can only be obtained in 50% yield, since the enzyme accepts only the α-amino acid amide as a substrate. In this process, if α-amino acid amide racemase could be used together with α-stereospecific hydrolases, the remaining α-amino acid amide would be racemized and the α-amino acid amide formed could be hydrolyzed by α-stereospecific hydrolases, resulting in the production of optically active α-amino acid in 100% theoretical yield. It would also be possible to synthesize optically active l-amino acids in 100% yield, when amino acid amide racemase is used with α-stereospecific hydrolases.

Knowing the unique properties of ACL racemase which specifically acts on neutral cyclic amides but not on amino acids, we were prompted to examine the detailed catalytic activity of ACL racemase on amino acid amides. We synthesized ACL racemase gene of Agrobacterium rhizogenes According to the method of Bremer et al. [23] Amino acid methylesters were prepared from the corresponding amino acids with thionyl chloride in methanol at −20 ºC. Amino acid amides were then obtained by ammonolysis of the corresponding methylesters with dry NH3 gas in methanol at room temperature. 1H and 13C-NMR spectra were recorded by JEOL LA-400 spectrometers (Tokyo, Japan) with tetramethylsilane as an internal standard.

2. Enzyme assay

The standard reaction mixture (100 μl) for assaying the ACL racemase activity contained 100 mM potassium phosphate buffer (pH 7.0), 2 μM pyridoxal phosphate (PLP) and 100 mM d-ACL. The reaction was performed at 30 ºC for 10 min and stopped by the addition of 20 μl 2N HClO4. ACL racemase activity toward d-ACL was determined by measuring the amount of d-ACL formed. The amount of d-ACL formed was determined with a high-performance liquid chromatography (HPLC) equipped with a Crownpak CR (+) column at a flow rate of 0.6 ml/min, using the solvent system of 60 mM HClO4. Absorbance of the eluate was monitored at 200 nm. One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of d-ACL from l-ACL per minute.

2.5. Synthesis of ACL racemase gene from A. obae

The ACL racemase gene from A. obae is composed of 1305 nucleotides (435 amino acid residues) [24]. Forty-six base changes in the amino acids coding region were introduced in the ACL racemase...
oligonucleotides (26 nt × 50 nt, 10 nt × 49 nt, 2 nt × 48 nt, 4 nt × 47 nt, 2 nt × 46 nt, 2 nt × 40 nt) were designed on the basis of the nucleotide sequence of the ACL racemase gene from \textit{A. obae} [25]. Forty-six oligonucleotides configured complementary oligonucleotides, which will overlap by 20 nt (Fig. 1). EcoRI and SphI sites were introduced into the flanking region of the ACL racemase gene by using two primers (ACL1 and ACL46 oligonucleotides): ACL1 primer, 5’.

\begin{verbatim}
A1 5’ - gcggtagttaccggaagaggcaagtccatgatgacagcgtg -3’
A2 5’ - cctcagggtagttaccggaagaggcaagtccatgatgacagcgtg -3’
A3 5’ - gcaatgggacctgggaagaggcaagtccatgatgacagcgtg -3’
A4 5’ - cttcagggtagttaccggaagaggcaagtccatgatgacagcgtg -3’
A5 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A6 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A7 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A8 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A9 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A10 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A11 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A12 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A13 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A14 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A15 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A16 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A17 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A18 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A19 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A20 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A21 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A22 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A23 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A24 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A25 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A26 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A27 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A28 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A29 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A30 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A31 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A32 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A33 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A34 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A35 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A36 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A37 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A38 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A39 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A40 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A41 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A42 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A43 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A44 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A45 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
A46 5’ - gcgcctggtctaccggaagaggcaagtccatgatgacagcgtg -3’
\end{verbatim}

Fig. 1. Oligonucleotides synthesized based on the nucleotide sequence of ACL racemase gene from \textit{A. obae}. 

E. coli JM109 harboring pACL60 was subcultured at 37°C for 12 h in a test tube containing 5 ml LB medium supplemented with ampicillin. The subculture was then inoculated into a 2 l shaking flask containing 500 ml LB medium supplemented with 80 μg/ml ampicillin and 0.5 mM IPTG. After 8 h incubation at 37°C with reciprocal shaking, the cells were harvested by centrifugation at 8000 × g for 5 min at 4°C and washed with 0.85% NaCl. The buffer used throughout this study was 10 mM KPB, pH 7.0, containing 250 mM sucrose, 2 μM PLP and 5 mM 2-mercaptoethanol. Washed cells from 10 l culture were resuspended in 10 mM buffer and disrupted by sonication for 10 min (19 kHz; Insonator model 201 M; Kubota, Tokyo, Japan). For the removal of intact cells and cell debris, the lysate was centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was heated at 60°C for 10 min followed by centrifugation [26]. The supernatant was applied to a DEAE-Toyopearl 650M column equilibrated with 10 mM buffer. After the column was washed thoroughly with 10 mM buffer, the enzyme was eluted by a linear gradient of 0 to 300 mM KCl in 10 mM buffer. The active fractions were combined and then brought to 30% (NH₄)₂SO₄ saturation and added to a Butyl-Toyopearl 650M column equilibrated with 10 mM buffer 30% saturated (NH₄)₂SO₄. After the column had been washed with the same buffer, the enzyme was eluted by a linear gradient of 30–0% (NH₄)₂SO₄ in 10 mM buffer. The active fractions were combined, concentrated by a Centricon YM-10, and applied to a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech, Sweden) at 0.4 ml/min, and the active fractions were collected and concentrated by a Centricon YM-10. The protein concentration was estimated by an absorbance at 280 nm, and determined by the method of Bradford using the dye reagent supplied by Bio-Rad. Bovine serum albumin was used as standard.

2.8. Substrate specificity

The substrate specificity was measured in a reaction mixture containing 2 μM PLP, 100 mM KPB (pH 7.0), 100 mM substrate, and an appropriate amount of enzyme, and the reaction mixture (2 ml) was incubated at 30°C. The reaction was stopped by the addition of HClO₄. The concentration of the products was determined with HPLC.
3. Results

3.1. Synthesis of ACL racemase gene from A. obae, cloning, and construction of pACL

The ACL racemase gene consists of 1305 bp that encodes a protein of 435 amino acid residues [24]. Forty-six oligonucleotides were synthesized on the basis of the nucleotide sequence of the ACL racemase gene from *A. obae*. The *EcoRI-SphI* fragment of the amplified PCR product with a size of 1380 bp was extracted from agarose gel and ligated into pUC18, and then used to transform *E. coli* JM109 cells. *E. coli* JM109/pACL60 showed ACL racemase activity. Plasmid pACL60 containing the fragment coding for the ACL racemase was extracted from *E. coli* JM109/pACL60, and the DNA sequence of the inserted fragment was determined to be identical with the reported sequence (Fig. 2) [24].

3.2. Substrate specificity of ACL racemase

ACL racemase was purified from the cell-free extract of *E. coli* JM109/pACL60 in 16% yield, by a procedure involving heat treatment, and chromatography on DEAE-Toyopearl 650M, Butyl-Toyopearl 650M, Superdex 200 HR 10/30, and MonoQ HR 5/5 columns. The final preparation gave a single band on SDS-PAGE. After purification, 4.2 mg of ACL...
Substrate specificity of ACL racemase

The enzyme activity was assayed as described in Section 2. The following compounds were not a substrate for the ACL racemase: 1-arginine amide, L-histidine amide, 1-tryptophan amide, L-tryptophan amide, L-valine amide, L-ornithine amide, L-glutamate amide, L-glutamine amide, L-serine amide, L-alanine amide, L-asparagine amide, L-glutamic acid, serine amide, and phenylalanine amide, it was 2.1, 1.7, 1.7, 1.5, 1.3, 0.94, 0.47, and 0.052%, respectively.

No racemizing activity was observed with α-H-amino acids, dipeptides containing alanine, and alanine methylester as described in the legend of Table 2.

3.3. Kinetic characterization of ACL racemase

Kinetic parameters for the D- and L-isomers of two amino acid amides (2-aminobutyric acid amide and alanine amide) and L-ACL were calculated from Lineweaver–Burk plots (Table 3). The \( K_a \) and \( K_m \) values for L-ACL were calculated to be 10.1 mM and 360 S\(^{-1}\), respectively.

\[ K_{cat} = \frac{V_{max}}{K_m} \]

\[ K_m = \frac{1}{V_{max} \cdot K_{cat}} \]

\[ V_{max} = \frac{1}{K_{cat} \cdot K_m} \]

\[ K_{cat} = \frac{1}{K_m} \]

Fig. 3 shows the time course of the racemization reaction of L-alanine amide and L-asparagine amide using ACL racemase. The reaction mixture contained 2 μM PLF, 100 mM KPB (pH 7.0), 40 mM substrate, and ACL racemase, and the reaction mixture was incubated at 30 °C, and controls were run without enzyme at the same condition. After 100 min, 2-aminobutyric acid amide and L-asparagine amide were calculated to be 40, 13, 9.8, and 7.2 S\(^{-1}\), respectively. The \( K_a \) values for the D- and L-2-aminobutyric acid amide and D- and L-alanine amide were calculated to be 40, 13, 9.8, and 7.2 S\(^{-1}\), respectively.

The enzyme activity was assayed as described in Section 2. The initial velocities were determined, and the steady-state kinetic parameters were calculated by using Lineweaver–Burk plots.

### Table 1

Summary of purification of ACL racemase from *E. coli* JM109/pACL60

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>1200</td>
<td>9100</td>
<td>7.60</td>
<td>100</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>210</td>
<td>8600</td>
<td>41.0</td>
<td>95</td>
</tr>
<tr>
<td>DEAE-Toyopearl 650M</td>
<td>150</td>
<td>7700</td>
<td>53.0</td>
<td>85</td>
</tr>
<tr>
<td>Butyl-Toyopearl 650M</td>
<td>53</td>
<td>4000</td>
<td>76.0</td>
<td>44</td>
</tr>
<tr>
<td>SUPeroxide 200HR 10/30</td>
<td>35</td>
<td>2800</td>
<td>80.0</td>
<td>31</td>
</tr>
<tr>
<td>MonoQ HR 5/5</td>
<td>4.2</td>
<td>1500</td>
<td>350</td>
<td>16</td>
</tr>
</tbody>
</table>

### Table 2

Substrate specificity of ACL racemase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-α-Amino-ε-caprolactam</td>
<td>100</td>
</tr>
<tr>
<td>L-2-Aminobutyric acid amide</td>
<td>2.7</td>
</tr>
<tr>
<td>L-Alanine amide</td>
<td>2.1</td>
</tr>
<tr>
<td>L-Threonine amide</td>
<td>1.7</td>
</tr>
<tr>
<td>L-Norvaline amide</td>
<td>1.3</td>
</tr>
<tr>
<td>L-Norleucine amide</td>
<td>1.3</td>
</tr>
<tr>
<td>L-Leucine amide</td>
<td>1.3</td>
</tr>
<tr>
<td>L-Methionine amide</td>
<td>1.3</td>
</tr>
<tr>
<td>L-Serine amide</td>
<td>0.94</td>
</tr>
<tr>
<td>L-Phenylalanine amide</td>
<td>0.052</td>
</tr>
</tbody>
</table>

The enzyme activity was assayed as described in Section 2. The following compounds were not a substrate for the ACL racemase: L-arginine amide, L-lysine amide, L-tryptophan amide, L-tryptophanamide, L-valine amide, L-ornithine amide, L-glutamate amide, L-glutamine amide, L-serine amide, L-alanine amide, L-asparagine amide, L-glutamic acid, serine amide, and phenylalanine amide, it was 2.1, 1.7, 1.7, 1.5, 1.3, 0.94, 0.47, and 0.052%, respectively.

### Table 3

Kinetic parameters for ACL racemase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_{cat} ) (S(^{-1}))</th>
<th>( K_m ) (μM)</th>
<th>( K_{cat}/K_m ) (μM S(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-α-Amino-ε-caprolactam</td>
<td>360</td>
<td>10.1</td>
<td>35.6</td>
</tr>
<tr>
<td>L-2-Aminobutyric acid amide</td>
<td>13</td>
<td>1.1</td>
<td>11.8</td>
</tr>
<tr>
<td>L-Alanine amide</td>
<td>7.2</td>
<td>2.5</td>
<td>2.88</td>
</tr>
<tr>
<td>L-Asparagine amide</td>
<td>9.8</td>
<td>3.6</td>
<td>2.88</td>
</tr>
</tbody>
</table>

The enzyme activity was assayed as described in Section 2. The initial velocities were determined, and the steady-state kinetic parameters were calculated by using Lineweaver–Burk plots.
amidase was completely racemized by the enzyme, and L-
alanine amidase was also completely racemized in 120 min. These facts clearly show that linear α-H-amino acid amidases can be racemized by ACL racemase.

4. Discussion

Many different kinds of racemases acting on amino acids and hydroxyl acids, etc. have been reported to occur in some bacteria, however, it is generally accepted that none of them acts on amino acid amidases. As for amino acid racemases, alanine racemase from S. typhimurium [2], glutamate racemase from Pedococcus pentonaceus [3], and asparagine racemase from Streptococcus faecalis [8] have very high specificities for the amino acid amidases. Threonine racemase from P. putida [4], arginine racemase from Pseudomonas graveolens [5], and amino acid racemase from P. putida [6] and Aeromonas punctata [7] show rather broader substrate specificities on amino acids.

α-Amino-ε-caprolactam is a heterocyclic compound used as the substrate for the enzymatic production of L-lysine in a Japanese chemical company, Toray Industries, Inc.: t-ACL is hydrolyzed to form L-lysine by t-ACL hydrolase of C. laurentii, and the unreacted t-isomer is racemized by ACL racemase of A. obtace [11]. Soda et al. reported that ACL racemase from A. obtace acts only on cyclic amino lactams such as ACL, α-amino-β-valerolactam [27,28] and α-amino-3-thio-
ε-caprolactam [13]. The properties of the ACL racemase hitherto known and the results of this study are summarized in Table 4 [9,10,12,13,26–29]. There has been no report that ACL racemase shows an activity towards amino acid amidases.

We predicted that ACL racemase would catalyze the racemization of amino acid amidases as well as cyclic amino lactam compounds, based on a consideration in the structural similarity of ACL to simple amino acid amidases: ACL has a free amino group on the α-position. Indeed, we have shown in this paper for the first time that the substrate specificity of ACL is rather wide and it acts on several amino acid amidases.

Recently, the genetic information has been tremendously expanded and the functions of the proteins are assumed and discussed by homology comparisons of their primary and higher structures. However, it goes without saying that their functions are not fully understood by these methods, until they are proven by experiments. It is one of the surest ways to express the gene and prove their functions by an actual experiment as we did here, if one really would like to use the genetic resources.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity (U/mg)</td>
<td>380 [26]</td>
</tr>
<tr>
<td>Molecular weight</td>
<td></td>
</tr>
<tr>
<td>Gel filtration</td>
<td>51000 [12]</td>
</tr>
<tr>
<td>Gene sequencing</td>
<td>45568 [29]</td>
</tr>
<tr>
<td>No. of subunits</td>
<td>Monomer [12]</td>
</tr>
<tr>
<td>pH optimum for</td>
<td></td>
</tr>
<tr>
<td>t-ACL</td>
<td>8.8 [12]</td>
</tr>
<tr>
<td>t-ACL</td>
<td>8.8 [12]</td>
</tr>
<tr>
<td>t-α-amino-β-valerolactam</td>
<td>7.0 [10]</td>
</tr>
<tr>
<td>Inhibitor</td>
<td></td>
</tr>
</tbody>
</table>
| Gly [12], Ala [12], Gln [12], Pro [12], Lys [12], L-Arg [12], L-His [12], L-Lys [12], L-Met [12], L-Orn [12], L-Phe [28], L-Trp [28], L-Lys [28], t-Orn [28], t-α-amino-
β-valerolactam, t-α-amino-
β-valerolactam, α-amino-
β-valerolactam, α-amino-
β-valerolactam, α-amino-
β-valerolactam, α-amino-
β-valerolactam, α-amino-
β-valerolactam, α-amino-
β-valerolactam, α-amino-
β-valerolactam, α-amino-
β-valerolactam, α-amino-
β-valerolactam, α-amino-
β-valerolactam, α-amino-
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β-valerolactam, α-amino-
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β-valerolactam, α-amino-
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Construction of a synthetic gene using assembly PCR provides a fast way to clone and express such genes in E. coli without isolating the target gene from biological materials. We have succeeded in the synthesis of the ACL racemase gene from A. obae by assembly PCR of oligonucleotides [25], cloning and expression of the gene, purification of the E. coli enzyme from [25], cloning and expression of the gene, purification of the enzyme from E. coli JM109/pACL60, and determination of the racemization reaction toward α-H-amino acid amides as substrates. The expression level of the enzyme by the E. coli transformant was rather low, although we did not optimize the culture and its induction conditions. The purification procedure of the enzyme may be simplified by adding His-Tag to the protein.

We reported the newly discovered activity of ACL racemase towards amino acid amides with detailed kinetic parameters. This is one of the first reports on amino acid racemase towards amino acid amides with detailed kinetic parameters. The expression level of the enzyme by the E. coli transformant was rather low, although we did not optimize the culture and its induction conditions. The purification procedure of the enzyme may be simplified by adding His-Tag to the protein.

We have succeeded in the synthesis of the ACL racemase without isolating the target gene from biological materials. We here showed that the racemization reaction of 1-2-amino acid amides and alanine amide were compared, the $K_{cat}/K_{m}$ values for 2-amino acid amide (11.8 S$^{-1}$ mM$^{-1}$ for L-enantiomer, and 11.4 for D-enantiomer) were higher than those for alanine amide (2.88 for L-enantiomer and 2.88 for D-enantiomer). The substrate specificity showed the preference of the enzyme for substrates with shorter aliphatic side chain and the catalytic efficiencies for 2-amino butyric acid amide and alanine amide were 8.1–33% that for ACL. The low $K_{m}$ values towards these substrates contributed much for the relatively high catalytic efficiencies. We here showed that the racemization reaction of 1L-2-amino acid amide and alanine amide progressed completely. If the ACL racemase will be used together with a $\Delta$-stereospecific hydrolase, it would be possible to synthesize $\Delta$-amino acids in 100% theoretical yield [14]. With an L-stereospecific hydrolase, this will result in high yield production of optically active amino acid without loss of substrate, thereby also reducing the production costs. The results of the dynamic kinetic resolution of amino acid amides to form optically active amino acid using ALC racemase and stereospecific amino acid amide hydrolases will be reported elsewhere.

References


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