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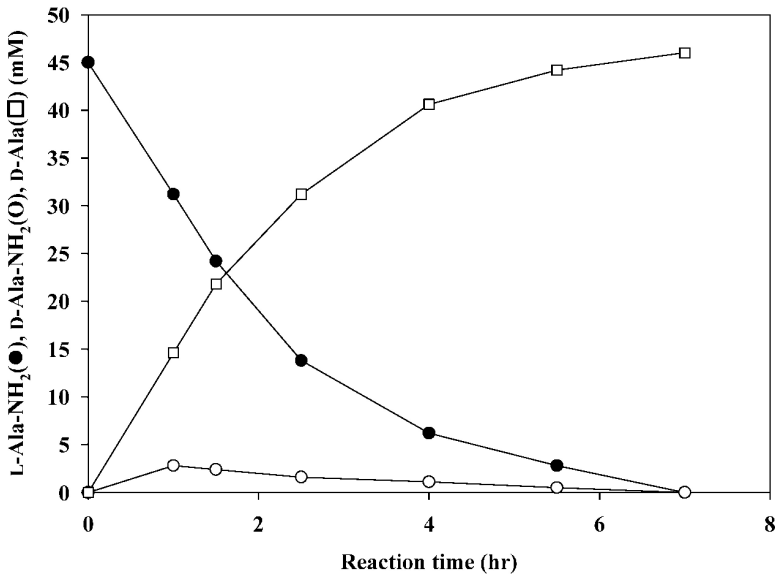

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Dynamic Kinetic Resolution of Amino Acid Amide Catalyzed by D-Aminopeptidase and α-Amino-ϵ-caprolactam Racemase

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D-Amino acids and their derivatives are important commercial products with applications in feed supplements, medicine, cosmetics, and as building blocks for compounds with biological or physiological activities. Many enzymes are hitherto used for the production of D-amino acids.[1] Recent examples include the dynamic kinetic resolution of hydantoins in an industrial scale with hydantoinase (EC 3. 5. 2. 2) in combination with *N*-carbamoyl-D-amino acid amidohydrolase (EC 3. 5. 1. 77),[2] and of racemic *N*-acyl-amino acids with *N*-acyl-D-amino acid amidohydrolase (EC 3. 5. 1. 14)[3] in combination with *N*-acyl-amino acid racemase (EC 4. 2. 1. -).[4] Asymmetric synthesis from α-keto acids has been also achieved with D-amino acid aminotransferase (EC 2. 6. 1. 21) and other enzymes.[5]

We have reported the discoveries, properties, and structures of new enzymes, D-aminopeptidase (DAP; EC 3. 4. 11. 19), D-amino acid amidase (EC 3. 5. 1. -), and alkaline D-peptidase from *Ochrobactrum anthropi* C1-38, *O. anthropi* SV3, and *Bacillus cereus* DF4-B, respectively.[6−10] These D-stereospecific hydrolases can be applied to kinetic resolution of racemic amino acid amides to yield D-amino acids. By this route, D-amino acid may be prepared in three steps from an aldehyde.[11] The amino acids can only be obtained in 50% yield since the enzyme accepts one of the D-enantiomers as a substrate. If amino acid amide racemase were used together with these D-stereospecific hydrolases, the remaining L-amino acid amide could be racemized and DL-amino acid amide would be completely hydrolyzed to form D-amino acid. It would also be possible to synthesize L-amino acids when amino acid amide racemase is used with L-stereospecific hydrolases.

We discovered amino acid amide racemizing activity in α-amino-ϵ-caprolactam (ACL) racemase (EC 5. 1. 1. 15) from *Achromobacter obae*. Soda et al. reported that the substrates for ACL racemase are only ACL, α-amino-δ-valerolactam, and α-amino-β-thia-ϵ-caprolactam, but the enzyme does not act on tryptophan amide or leucine amide.[12−16] After completing our studies, we noticed that α-H-amino acid amide racemizing activities from *Agrobacterium rhizogenes*, *Arthrobacter nicotianae*, and *Ochrobactrum anthropi*, and some of the gene sequences were described in a recent patent.[17] However, it does not mention that ACL racemase catalyzes the racemization of amino acid amides. We here demonstrate the result of a construction of an efficient system to produce D-amino acid from L-amino acid amide by a combination of ACL racemase and D-aminopeptidase. This is the first report of the enzymatic synthesis of stoichiometric amounts of D-amino acid from L-amino acid amide by a combination of a new amino acid racemizing enzyme and D-stereospecific amino acid amide hydrolase.

Forty-six oligonucleotides were designed on the basis of nucleotide sequence of *A. obae* ACL racemase gene, the open reading frame of which is composed of 1305 nucleotides (435 amino acid residues). The oligonucleotides were configured complementary to overlap by 20 nucleotides,[18,19] combined, and assembled in PCR. The ACL racemase gene was amplified by using combinations of the two outside primers, the first and the last oligonucleotide (ACL1 and ACL46 oligonucleotides). *Eco*RI-*Sph*I fragment of the amplified PCR product in the size of 1380 bp was extracted from agarose gel and ligated with pUC18, and then used to transform *E. coli* JM109 cells. A transformant *E. coli* JM109/pACL60 showed the activity of ACL racemase. The plasmid pACL60 was extracted from *E. coli* JM109/pACL60, and the inserted fragment was determined to be identical with the reported DNA sequence.[19]

The ACL racemase was purified from *E. coli* JM109/pACL60 by heat treatment and DEAE-Toyopearl 650M, Butyl-Toyopearl 650M, Superdex 200 HR, and MonoQ HR column chromatographies.[20] The final preparation gave a single band on SDS−PAGE. The substrate specificity of the ACL racemase was examined with cyclic amino caprolactams, together with new substrates amino acid amides and peptides.[21] The enzyme acted broadly on various amino acid amides, such as L-2-aminobutyric acid amide, L-alanine amide, L-threonine amide, L-norvaline amide, L-norleucine amide, L-leucine amide, L-methionine amide, L-serine amide, and L-phenylalanine amide.[22] Kinetic parameters for the two D- and L-isomers of amino acid amides (alanine amide and 2-aminobutyric acid amide) and L-ACL were obtained from Lineweaver−Burk plots. The K_m values for L-ACL and D- and L-2-aminobutyric acid amide and D- and L-alanine amide were calculated to be 10, 3.5, 1.1, 3.4, and 2.5 mM, respectively. The V_{max} values toward L-ACL and D- and L-2-aminobutyric acid amide and D- and L-alanine amide were shown to be 440, 49, 16, 12, and 8.8 μmol/(min mg protein), respectively. Figure 1 shows the time course of racemization reaction of 40 mM L-alanine amide using the ACL racemase. After 2 h, L-alanine amide was completely racemized by the reaction. L-2-Aminobutyric acid amide was also completely racemized by the same reaction condition (data not shown).

These results clearly show that amino acid amides were racemized by ACL racemase. On the other hand, no racemizing activity was observed with the dipeptides or amino acid derivatives, such as L-alanyl-D-alanine, D-alanyl-L-alanine, L-alanylglycine, L-phenylglycine, or L-alanine methylester.

We previously reported the D-stereospecific hydrolysis of D-amino acid amides by DAP from *O. anthropi* C1-38. DAP gene (*dap*) of *O. anthropi* C1-38 was cloned in *E. coli* JM109, and an expression plasmid, pDAP1, was constructed. DAP was purified from *E. coli* JM109/pDAP1 harboring the *dap* gene for D-aminopeptidase as described previously.[6,23] The final preparation gave a single band on SDS−PAGE.

The enzymatic production of D-amino acid from L-amino acid amide has been demonstrated by use of ACL racemase from *A. obae* and DAP from *O. anthropi* C1-38 by one-pot reaction. When DAP and ACL racemase were used at the same time, 45 mM L-alanine amide was completely converted to D-alanine after 7 h and the final yield of D-amino acid was more than 99.7%.

These results show that it has been possible to construct an efficient system to produce D-amino acid from DL-amino acid amide by the ACL racemase and D-stereospecific hydrolase. The strategy from DL-amino acid amides to D-amino acids could be applicable

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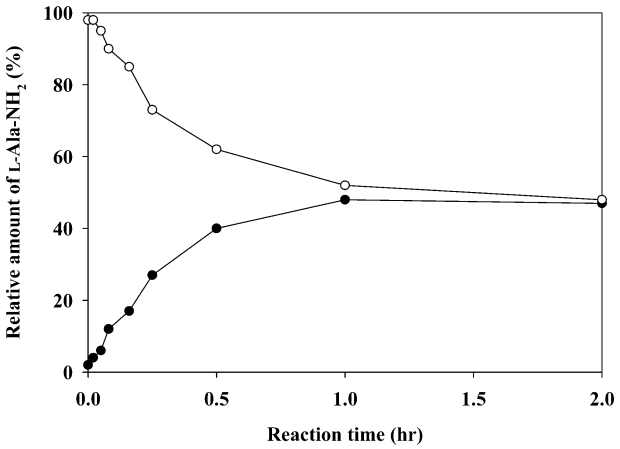

Figure 1. Enzymatic racemization of D-alanine amide and L-alanine amide by ACL racemase. The reaction mixture (2.0 mL) contained 200 μmol of KPB (pH 7.0), 4 nmol pyridoxal phosphate, 80 μmol of L-alanine amide, and 2.7 nmol (136 μg, 47.6 units) of ACL racemase at 30 °C and pH 7.0. The reaction was followed by HPLC at the point shown in the graphic.[24] Symbols: (●), D-alanine amide; (○), L-alanine amide.

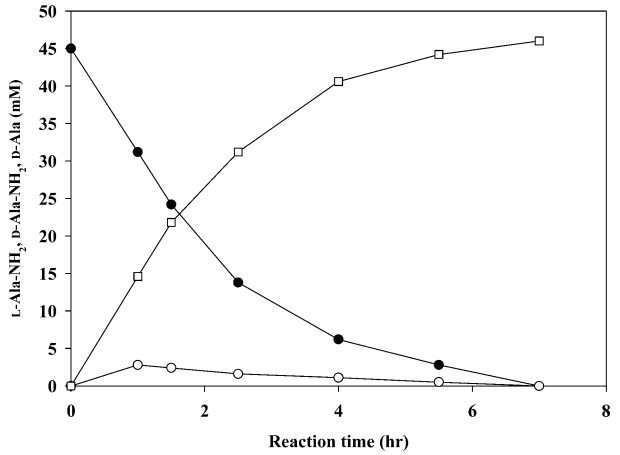

Figure 2. Production of D-alanine from L-alanine amide. The reaction mixture (2.0 mL) containing 200 μmol of KPB (pH 7.0), 4 nmol pyridoxal phosphate, 90 μmol of L-alanine amide, 1.35 nmol (68 μg, 23.8 units) of ACL racemase, and 46 pmol (5.4 μg, 2.2 units) of DAP at 30 °C and pH 7.0.[24−26] Symbols: (○), D-alanine amide; (●), L-alanine amide; (□), D-alanine.

to many D-amino acids. The production of L-amino acid could also be performed in our system that is fed with DL-amino acid amides, containing ACL racemase and L-stereospecific hydrolase.

Supporting Information Available: The SDS−PAGE of purified ACL racemase and DAP are shown in Figure S1 (PDF). HPLC analytical data for all compounds are in Figure S2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) The standard reaction mixture for assaying the amino acid amides' racemase activity contained 100 mM potassium phosphate buffer (KPB), pH 7.0, 2 μM pyridoxal phosphate, and 100 mM L-amino acid amide. The amount of D-amino acid amide was determined by high-performance liquid chromatography (HPLC) equipped with a Crownpak CR (+) column (Daicel Chemical Industries, Ltd., Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of D-ACL from L-ACL per minute.
(22) The relative specific activities (at 100 mM each) to ACL (350 U/mg at 100 mM L-ACL) were achieved with L-2-aminobutyric acid amide (2.7%), alanine amide (2.1%), threonine amide (1.7%), norvaline amide (1.7%), norleucine amide (1.5%), leucine amide (1.3%), methionine amide (0.94%), serine amide (0.47%), and phenylalanine amide (0.052%).
(23) The enzyme activity was assayed by the formation of *p*-nitroaniline from D-alanine-*p*-nitroanilide as follows. A reaction mixture contained 50 mM KPB (pH 7.0), 50 mM D-alanine-*p*-nitroanilide, and an appropriate amount of the enzyme. The enzyme activity was monitored by the change in absorbance at 405 nm with a Hitachi U-3210 spectrophotometer. A linear change in absorbance for 1 min was employed in the kinetic study. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of *p*-nitroaniline from D-alanine-*p*-nitroanilide per minute by using an extinction coefficient of *p*-nitroaniline, 7620 M^{-1} cm^{-1}.
(24) The amounts of DL-alanine amide and DL-alanine were determined with a HPLC equipped with a Crownpak CR (+) column at a flow rate of 0.6 mL/min, using the solvent system of 60 mM $HClO_4$. Absorbance of the eluate was monitored at 200 nm (Supporting Information, Figure S2).
(25) The identity of D-alanine formed by the ACL racemase, and DAP of L-alanine amide was confirmed by its isolation. The reaction mixture contained 1.0 mmol (124 mg) of L-alanine amide−HCl, 1.0 mmol of KPB (pH 7.0), 20 nmol pyridoxal phosphate, 6.75 nmol (340 μg) of ACL racemase, and 230 pmol (27 μg) of DAP. After the mixture was incubated at 30 °C for 8 h, D-alanine formed in the mixture was isolated by a procedure involving deproteinization by trichloroacetic acid and column chromatography on a Dowex-X8 (H^+) column. The isolated D-alanine (0.92 mmol, 82 mg) was recrystallized from water−methanol−isopropyl alcohol−ether. The optical purity of the isolated D-alanine was more than 99.9%.
(26) When DL-alanine amide (45 mM) was used as a substrate in the same reaction condition, DL-alanine amide was completely converted to D-alanine.

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