New enzymatic synthetic methods of chiral amino acids and amines

Kazuyuki Yasukawa

March, 2014

Contents

Introduction

Chiral compounds are very important building blocks in the synthesis of biologically active compounds. These building blocks include nitrogen and oxygen containing compound such as amine or amino acid. Many chemical or enzymatic synthetic methods have been described. Today, both the academia and the industry focus on the efficient enzymatic synthesis of chiral compounds as a hot topic of research, especially for the development of sustainable technologies for the production of chiral compounds. Enzymatic synthesis can be used not only for syntheses under mild conditions but also for the introduction of a chiral center in a molecule.

The works presented in this thesis introduce a new enzymatic synthetic methods of chiral amino acids and amines.

Natural and unnatural chiral α-amino acids are extensively used for pharmaceutical, animal feed, and artificial sweetener. Fermentation methods have been successful in the production of (*S*)-amino acids such as lysine, phenylalanine, tryptophan, threonine, arginine, histidine, isoleucine, serine and valine in industrial scale.[1] On the other hand, a few proteinogenic (*S*)-amino acids and non-natural amino acids are produced by enzymatic method. Enzymatic synthesis of chiral α-amino acid involves the following: (i) Asymmetric reductive amination of α-keto acid, for example, the synthesis of (*S*)-amino acid from α-keto acid with NAD(P)-dependent L-amino acid dehydrogenase with cofactor regeneration.[2,3] The synthesis of (*R*)-amino acid with D-amino acid dehydrogenase created by directed evolution from *meso*-2,6-D-diaminopimelic acid dehydrogenase.[4] (ii) Asymmetric addition of ammonia to α, β-unsaturated carboxylic acid. (*R*)-amino acid can

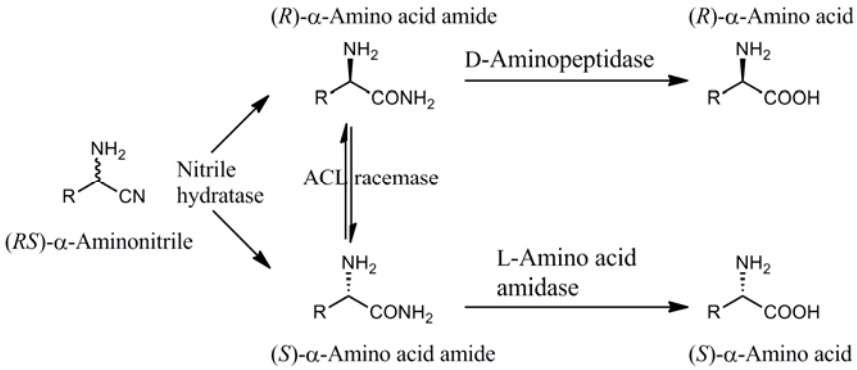
be synthesized using D-amino acid aminotransferase from other amino acid and α-keto acid, although it gives a mixture of four compounds. Ammonia lyase and aminomutase were mainly uesd to produce (*S*)-α-amino acid and (*R*)-β-amino acid, respectively.[5] Especially, aspartase and phenylalanine ammonia lyase catalyzed addition of ammonia to fumaric acid and cinnamic acid, and directly converted the substrates to (*S*)-aspartate and (*S*)-phenylalanine, respectively which were required in large quantities for the aspartame production. Methylaspartase catalyzed synthesis of methylaspartates reported by Asano et al.[6] (iii) Kinetic resolution (KR) by hydrolysis of racemic amino acid derivatives using stereoselective lipase, acylase, amidase, hydantoinase and carbamoylase is traditional and easy to use method. However, the theoretical yield of product is limited to 50% in the KR.[7] KR of α-aminonitriles using nitrilase[8-12] and dynamic kinetic resolution (DKR) are the examples of the third category.[13] DKR would be one of the most powerful and elegant methods for efficient synthesis of one enantiomer from racemic starting materials. Therefore, DKR has attracted increasing interests from both the industrial and the academic sides. For instance, optically pure α-amino acids were produced from α-amino-ε-caprolactam (ACL), *N*-acyl-α-amino acid, and 5-monosubstitued hydantoin compounds to be racemized and hydrolyzed by racemase and stereoselective hydrolase(s) in one pot reaction.[13]

Asano et al. focused on α-amino acid amides as starting compounds for chiral α-amino acid synthesis by DKR, because the substrate can be easily obtained by hydrolysis of α-aminonitrile which is prepared by Strecker synthesis. They have been studying many (*R*)- or (*S*)-stereoselective amino acid amide hydrolases since the information had been limited

on enzymes acting on α-amino acid amides and have utilized *R*-stereoselective amino acid amidases such as D-aminopeptidase, D-amino acid amidase, alkaline D-peptidase, and *R*-amidase in the KR of amino acid amides.[7] *S*-Stereoselective amino acid amide hydrolases such as L-amino acid amidase from *Pseudomonas azotoformans*[14] and *Brevundimonas diminuta*[15] have been characterized by Asano et al.

The purpose in this study is to obtain optically pure α-amino acids in theoretical yield with high enantiomeric excess by DKR of racemic α-aminonitriles. This DKR process consists of three steps: (i) hydration of both enantiomer of racemic α-aminonitrile by a non-stereoselective NHase (EC 4.2.1.84), (ii) stereoselective hydrolysis of resulting racemic α-amino acid amide by one of stereoselective amino acid amide hydrolases[14-18], and at the same time, (iii) racemization of remaining α-amino acid amide by ACL racemase (Scheme 1).[19-21] A non-stereoselective NHase is a key enzyme in this method.

Biotransformation of nitrile has been very successful industrial applications and has a great further potential in organic chemistry. Asano et al. have isolated various nitrile-degrading microorganisms[22-23], and first discovered and characterized NHase from *Rhodococcus rhodochrous* J-1 (formerly identified as *Arthrobacter* sp. J-1).[24-26] He also



Scheme 1. Dynamic kinetic resolution of α-aminonitrile to form chiral α-amino acid

discovered that *Pseudomonas chlororaphis* B23 accumulates huge quantities of amides from nitriles and is suitable for the industrial production of acrylamide from acrylonitrile.[22, 26] Moreover, nicotinamide and 5-cyanovaleramide are also industrially produced by NHase.[27-28]

Recently, Asano et al. have been successful in the enzymatic synthesis of nitriles from aldoximes by using the microbial enzyme aldoxime dehydratase (EC 4.99.1.5).[29-30] They isolated the enzyme for the first time and studied its enzymological properties[31], and found that the enzyme and nitrile-degrading enzymes such as NHase[32] and nitrilase[33] are linked genetically forming clusters[34-35], in the "aldoxime-nitrile pathway".

In the past, several NHase have been purified and characterized from various microorganisms.[24-26, 36-44] However, none of these has been explored in detail for their catalytic activity to hydrate α-aminonitriles. Most of research for the enantioselective NHase for α-aminonitrile has been done in whole cell systems rather than with purified NHase.[10-12, 45-47] Whole cell system is complicated by the presence of amidases that catalyze the hydrolysis of the product α-amino acid amides and their velocities are not measured, but only by yields of the products. Thus, in this study, the author reports the screening, purification, characterization of α-aminonitriles hydrating NHase, and cloning of its gene and its application to the DKR of α-aminonitriles to form chiral α-amino acids.

On the other hands, the efficient enzymatic synthesis of chiral amines as a building block in the fields of pharmaceuticals and agrochemicals has also been the focus of academia and industry. Lipase, *R,* or *S*-stereoselective transaminase has been mainly used to examine the enzymatic synthesis of chiral amines.[48-50] The deracemization of racemic

amine to form (*R*)-amine has been reported using *S*-stereoselective amine oxidase employing a chemical reductant.[51-56] Amine oxidases (AOx) catalyze the oxidative deamination of amine to form aldehyde, hydrogen peroxide, and ammonia via an imine intermediate. AOx can be classified into two groups based on the type of cofactor in the active site, namely Cu containing TPQ-dependent AOx[57] and flavin-dependent AOx [58]. These AOx preferentially oxidize simple straight chain primary amines such as butylamine, phenylethylamine, and dopamine rather than chiral amines at the alpha position such as α-methylbenzylamine (MBA).[59-60] Several studies have examined the actions of AOx on chiral (*S*)-amine.[51-56] Turner et al. reported engineered *S*-stereoselective flavin-dependent monoamine oxidase (MAO) variants from *Aspergillus niger* for the deracemization of racemic amines producing chiral primary, secondary, and tertiary amines.[51, 52, 55, 56] The catalytic activity of these mutants toward chiral primary amines was shown to be higher than that of simple primary amines. Leisch et al. successfully synthesized (*R*)-amine by deracemization using *S*-stereoselective cyclohexyl amine oxidase from *Brevibacterium oxydans* IH-35A.[53, 54] Kohler, et al. more recently synthesized secondary cyclic (*R*)-amines from their corresponding racemic amines or imine compounds by a cascade reaction with a metalloenzyme under mild conditions.[61] The reaction consisted of two steps; amine oxidation by mutant MAO and the reduction of imine with an artificial transfer-hydrogenase[62] instead of a harsh chemical reductant. However, *R*-stereoselective AOx suitable for deracemization have not yet been identified. TPQ-dependent AOx from *Escherichia coli* and *Klebsiella oxytoca* were shown to oxidize the *R*-enantiomer of amphetamine preferentially with moderate enantiomeric ratios (E =~15)[63], although they

are not suitable for the deracemizaion reaction because cofactor TPQ and intermediate imine formed a covalent bond. The purpose of the present study was to evolve flavin-dependent porcine kidney D-amino acid oxidase (pkDAO) into *R*-stereoselective AOx and apply it to the deracemization of racemic amines.

In this thesis, the author studied functional analysis of α-aminonitrile hydrolyzing enzyme and its application to chiral amino acids synthesis by dynamic kinetic resolution. Furthermore, development new *R*-stereoselective amine oxidase applicable to the production of (*S*)-amine by deracemization method was described.

In chapter I, the author described the isolation and characterization of non-stereoselective nitrile hydratase from α-aminonitrile hydrolyzing microorganism. He also developed new enzymatic synthesis of chiral amino acid by dynamic kinetic resolution.

In chapter II, the cloning and expression of nitrile hydratase gene in *Escherichia coli*, and its application for production chiral amino acids were carried out.

In chapter III, enzymatic synthesis of chiral phenylalanine derivatives by a dynamic kinetic resolution of corresponding amide and nitrile substrates with recombinant *E. coli* encoding NHase as well as *E. coli* co-expressing D-amino acid amidase and mutant ACL racemase was established.

In chapter IV, he described the evolving *R*-stereoselective amine oxidase from porcine kidney D-amino acid oxidase. He also established to enzymatic deracemization of racemic amine using engineered enzyme.

Chapter I

Characterization of non-stereoselective nitrile hydratase applicable to the production of chiral α-amino acid by one-pot three enzyme cascade

Five new bacterial strains having low-stereoselective nitrile hydratase (NHase) activity toward (*RS*)-α-aminobutyronitrile (ABN) were isolated from soil. Strain 71D, a bacterium producing high NHase activity with low stereoselectivity, was selected and identified as *Rhodococcus opacus*. NHase was purified 256-fold with a yield of 12.3% from the cell-free extract of *R. opacus* 71D. This enzyme was characterized as an $\alpha_2\beta_2$ hetero-tetramer with a native molecular weight of 119,000, composed of α- and β-subunits with molecular weights of 32,300 (β-subunit) and 27,200 (α-subunit), respectively. Optimum pH and temperature for the activity of the purified enzyme were 8.0 and 20°C, respectively. NHase from *R. opacus* 71D showed wide substrate specificity toward a variety of nitriles including α-aminonitrile, and aliphatic and aromatic nitriles. The specific activity of the purified enzyme was 1,040 U mg^{-1} protein with its K_m being 4.7 mM, toward (*RS*)- α-ABN. The enantiomeric ratio of several (*S*)-α-amino acid amides obtained with the purified enzyme were shown to be very low (*E*=1.0-2.1), as expected. Thus, purified NHase could be used as a bio-catalyst to hydrate racemic α-aminonitriles to corresponding racemic α-amino acid amides.

Experimental section

Materials.

(*RS*)-α-Aminobutyronitrile·1/2 H_2SO_4, (*RS*)-alaninonitrile·1/2 H_2SO_4, (*RS*)-valinonitrile·1/2 H_2SO_4, (*RS*)-leucinonitrile·1/2 H_2SO_4, (*RS*)-*tert*-leucinonitrile·1/2 H_2SO_4, (*RS*)-phenylalaninonitrile·1/2 H_2SO_4, and (*R*)-α-aminobutyric acid were obtained from Mitsubishi Gas Chemicals Co., Ltd. (Niigata, Japan) as gifts, or were synthesized in Laboratory of Enzyme Chemistry and Engineering, Toyama prefectural University. (*RS*)-Phenylglycinonitrile hydrochloride was purchased from Aldrich. DEAE-Toyopearl 650M and Butyl-Toyopearl 650M were obtained from Tosoh (Tokyo, Japan). MIGHTYSIL PR-18 GP column was from Kanto Chemical Co., Inc. (Tokyo, Japan). Gigapite was from Seikagaku Kogyo (Tokyo, Japan). Superdex 200 High-Load 26/60 and Macro-Prep Ceramic Hydroxyapatite Type 1 were from Amersham Bioscience (Uppsala, Sweden). Crown pak CR (+) column was from Daicel Chemical Industries, Ltd (Osaka, Japan). All other chemicals were from commercial sources.

Bacterial strain, plasmids, and culture conditions.

Rhodococcus opacus 71D isolated from soil, was cultivated at 30°C for 72 h with agitation at 96 rpm in the basal medium containing 0.2% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4·7H_2O$, 0.001% $CaCl_2$, 0.05% polypepton, 0.1% trace metals solution, and 0.3% butyronitrile. The trace metals solution contained the following salts in 1 L of water: 0.01 g $ZnSO_4·7H_2O$, 0.001 g $CuSO_4·5H_2O$, 0.001 g $MnSO_4·5H_2O$, 0.01 g $FeSO_4·7H_2O$, $Na_2MoO_4·2H_2O$, and $CoCl_2·6H_2O$.

Escherichia coli JM109/pACL60 was grown as described previously.[15, 19] *E. coli* JM109/pDAP1 was cultured at 37°C for 24 h in LB medium containing 80 μg/ml ampicillin and 0.5 mM IPTG.

Enzyme assay and definition of a unit of NHase activity.

The reaction mixture (total volume 1.0 ml) was composed of 50 mM potassium phosphate buffer (KPB), pH 7.0, 20 mM (*RS*)-α-aminobutyronitrile (ABN), 0.1 mM $CoCl_2$, and an appropriate amount of the enzyme. The enzyme was added to initiate the reaction. After incubation at 30°C, a sample (0.1 ml) was taken from the reaction mixture at several intervals. The reaction was stopped by adding 900 μl of 60 mM $HClO_4$. The amount of α-aminobutyramide (ABA-NH_2) formed in the reaction mixture was determined with a HPLC apparatus equipped with a Crown Pak CR (+) column (ϕ 0.4 x15 cm) at a flow rate of 0.3 ml min^{-1} using the solvent system of 60 mM $HClO_4$/10% MeOH.

One unit (U) of NHase activity was defined as the amount of bacterial cells or enzyme that catalyzed the formation of 1 μmol of α-ABA-NH_2 from α-ABN per min.

D-aminopeptidase (DAP).

DAP activity toward (*R*)-α-ABA-NH_2 was determined by measuring the amount of (*R*)-α-aminobutyric acid (ABA) formed. One unit (U) of DAP activity was defined as the amount of the enzyme that catalyzed the formation of 1 μmol of (*R*)-α-ABA from (*R*)-α-ABA-NH_2 per min.[16]

Preparation of purified ACL racemase.

E. coli JM109/pACL60 was subcultured at 37°C for 12 h in a test tube containing 5 ml of LB medium supplemented with 80 μg/ml ampicillin. The subculture was then transferred at 37°C for 12 h in 500 ml of LB medium supplemented with 80 μg/ml ampicillin and 0.5 mM IPTG. After 24 h, cells were harvested by centrifugation at 8,000 x *g* for 10 min at 4°C and washed with 0.9% NaCl. Cells were resuspended in 20 mM KPB (pH 7.0), containing 20 μM PLP, and disrupted by sonication for 10 min (19 kHz; Insonator model 201M; Kubota, Tokyo, Japan). To remove intact cells and cell debris, the lysate was centrifuged at 8,000 x *g* and 4°C for 15 min. The supernatant was heated at 60°C for 10 min followed by centrifugation to remove inactivated precipitates. The enzyme solution was applied to a DEAE-Toyopearl 650M column equilibrated with 10 mM KPB containing 20 μM PLP. After the column had been washed with 10 mM KPB including 20 μM PLP, the enzyme was eluted with a linear gradient of NaCl (0-500 mM) in the same buffer. The enzyme thus purified was dialyzed against 10 mM KPB containing 20 μM PLP. The enzyme solution was applied to a Mono Q 5/5 Column equilibrated with 10 mM KPB containing 20 μM PLP. After the column had been washed with 10 mM KPB containing 20 μM PLP, the enzyme was eluted with a linear gradient of NaCl (100-300 mM) in the same buffer. The enzyme thus purified was dialyzed against 10 mM KPB containing 20 μM PLP.

An activity assay was performed with a reaction mixture comprised of 50 mM KPB (pH 7.0), 100 mM (*R*)-α-ABA-NH_2, and 2 μM PLP. One unit (U) of ACL racemase activity was defined as the amount of the enzyme that catalyzed the formation of 1 μmol of (*S*)-α-ABA-NH_2 from (*R*)-α-ABA-NH_2 per min.

Purification of NHase from* R. opacus *71D.

R. opacus 71D was used as the source of the enzyme for purification. Enzyme purification was performed at 4°C unless otherwise stated. The KPB used in the purification process contained K_2HPO_4: KH_2PO_4 (pH 7.0), 0.1 mM $CoCl_2$, and 4 mM *n*-butyric acid. Cells were harvested by centrifugation at 15,000 x *g* for 5 min, washed by 10 mM KPB containing 0.9% NaCl, then washed by 10 mM KPB, and resuspended in the same buffer. For the preparation of cell-free extracts, cells were disrupted by ultrasonic disintegration twice for 10 min. The homogenate was centrifuged at 15,000 x *g* for 10 min and a supernatant was obtained. Solid ammonium sulfate to 30% saturation was added to the enzyme solution. After stirring, the precipitate was removed by centrifugation at 15,000 x g for 20 min and the supernatant was obtained. The supernatant was dialyzed against the same buffer. Solid ammonium sulfate was added to the enzyme solution to 30% (w/v) saturation and centrifuged at 15,000 x *g* for 10 min and a supernatant was obtained. The enzyme solution was applied to a Butyl-Toyoperl column (ϕ 2.8 x 8 cm) equilibrated with 10 mM KPB 30% saturated with ammonium sulfate. After washing the column with 10 mM KPB 20% saturated with ammonium sulfate, the enzyme was eluted with a 400 ml liner gradient of 20-0% saturation of ammonium sulfate. The enzyme thus purified was dialyzed against 10 mM KPB. The enzyme solution was applied to a DEAE-Toyopearl 650M column (ϕ 1.8 x 10 cm) equilibrated with 10 mM KPB. After the column had been washed with 10 mM KPB, the enzyme was eluted with a linear gradient of NaCl (0-500 mM) in the same buffer. The enzyme thus purified was dialyzed against 10 mM KPB.

The enzyme solution was applied to a GIGA-PITE column (ϕ 1.8 x 5 cm) equilibrated with 10 mM KPB. After washing the column with the same buffer, the enzyme was eluted with 50 ml liner gradient of 10-300 mM KPB. The enzyme thus purified was dialyzed against 10 mM KPB. The enzyme solution was applied to a Mono Q 5/5 Column equilibrated with 10 mM KPB. After the column had been washed with 10 mM KPB, the enzyme was eluted with a linear gradient of NaCl (200-400 mM) in the same buffer. The enzyme thus purified was dialyzed against 10 mM KPB. The enzyme solution was applied to a hydroxyapatite column (ϕ 1.8 x 2 cm) equilibrated with 10 mM KPB. After washing the column with the same buffer, the enzyme was eluted with 50 ml liner gradient of 10-300 mM KPB. The enzyme thus purified was dialyzed against 10 mM KPB. The dialyzed enzyme was used for characterization.

Conversion of (R)-α-aminobutyric acid from (RS)-α-aminobutyronitrile.

The identity of (*R*)-α-ABA formed by NHase, ACL racemase, and DAP was confirmed by its isolation. The reaction mixture (40 mL) contained 2.0 mmol KPB (pH 8.0), 4.0 mmol (*RS*)-α-ABN sulfuric acid salt (0.532 g), 80 nmol pyridoxal phosphate, 4.0 μmol $CoCl_2$, 120 U of NHase, 39 U of ACL racemase, and 93 U of DAP. After the mixture was incubated at 30°C for 10 h, (*R*)-α-ABA formed was isolated by Dowex-X8 column chromatography and recrystallized from water-methanol-ether. The optical purity of the isolated (*R*)-α-ABA was more than 99% ee with isolation yield 68% (0.28 g, 2.72 mmol); Optical rotations were measured on a SEPA-300 (Horiba, Ltd., Kyoto, Japan). $[\alpha]^{20}_D$ -19.7° (*c* 1.00, 5 mol dm^{-3} HCl) (ref. 64, $[\alpha]^{20}_D$ -20.5° (*c* 1.00, 5 mol dm^{-3} HCl)), ^1H NMR (D_2O,

400 MHz) δ: 3.60 (t, 1H, *J* = 5.8 Hz), 1.79 (dq, 2H, *J* = 5.8, 7.5 Hz), 0.87 (d, 3H, *J* = 7.5 Hz). ^{13}C NMR (400 MHz, D_2O) δ = 174.8, 55.8, 23.6, 8.5; MS (microTOF) *m*/*z*: calcd for $C_4H_{10}N_1O_2$ $[M+H]^+$ 104.0706; found, 104.0731.

RESULTS

Screening and isolation of non-stereoselective α-aminobutyronitrile-hydrolyzing microorganisms.

In the initial screening, microorganisms (64 strains) were isolated from soil as butyronitrile-utilizing microorganisms. The activity toward α-ABN was determined by thin layer chromatography (TLC) using the developing solvent *n*-butanol/acetic acid/water = 4/1/1, and α-ABA-NH_2 visualized with ninhydrin. Among the 64 isolates, 9 strains showed degradation activity toward α-ABN. The stereoselectivity of α-ABN degrading activity by these strains was determined by chiral HPLC. Enantiomeric ratios[65] of these strains for α-ABN were shown to be 1.2-2.7 (*S*). From these microorganisms, strain 71D was selected and identified as *R. opacus*, and was used for further investigations because of its very low stereoselectivity and highest activity toward α-ABN (Figure 1-1).

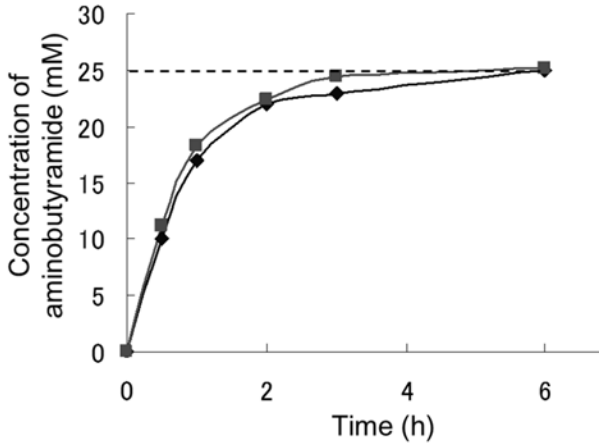


Figure 1-1. Time course of a-aminobutyramide formation catalyzed by acetone dried cells of *R. opacus* 71D.

The reaction mixture (1 mL) was composed of 50 mM Potassium phosphate buffer, pH 7.0, 50 mM (*RS*)-α-ABN, 0.1 mM $CoCl_2$ and acetone dried cells (1 mg).

Symbols: (*R*)-α-ABA-NH2 (♦) and (*S*)-α-ABA-NH2 (■).

Identification of non-stereoselective α-aminobutyronitrile-hydrolyzing microorganism, strain 71D.

The taxonomical characteristics of strain 71D are shown (Table 1-1a, 1-1b, 1-1c, 1-1d). In 16S rDNA sequence similarity, determination of partial sequences of the most variable region revealed 100% identical to that of *Rhodococcus opacus* or *Rhodococcus wratislaviensis* (the highest value). The taxonomical characteristics of the strain 71D indicate that it belongs to the genus *R. opacus*, because it is irregular rods (0.8 x 2.0-3.0 μm), Gram-positive, non-spore forming, non-motile, catalase-positive, oxidase-negative, acid fast-negative, utilization of carbon source: 4-aminobutyrate, D-ribose, tyramine are positive and L-rhamnose is negative (Table 1-2).

Table 1-1a. Taxonomical characteristics of the strains isolated from soil.

	71D
Grams stain	+
Shape	Rods
Color	Deep yellow
Size	0.8X2.0-3.0
Acid fast	-
Motility	-
Growth in air	+
Growth anaerobically	-
Catalase	+
Oxidase	-
Glucose	-
OF test (gas/acid)	(-/-)
16S rDNA	*R. opacus or R. wratislaviensis*

Table 1-1b. Acid and gas from sugars.

Various sugars (1%) were added to 10 ml medium containing 1% polypepton, 1% meat extract and 0.5% NaCl. Cultivation was carried out for 24 h at 30ºC with static culture.

	71D (gas/acid)
D-Glucose	(-/-)
Glycerol	(-/-)
D-Maltose	(-/-)
D-Xylose	(-/-)
D-Sorbitol	(-/-)
Sucroce	(-/-)
L-Arabinose	(-/-)
D-Fructose	(-/-)
D-Galactose	(-/-)
D-Mannose	(-/-)
Trehalose	(-/-)
D-Raffinose	(-/-)
D-Mannitol	(-/-)
Lactose	(-/-)
Starch potato	(-/-)

Table 1-1c. Taxonomical characteristics.

	71D
NO_2 and NO_3 reduction	-
Denitrification	-
MR test	-
VP test	-
Indole formation	-
H_2S formation	-
Starch hydrolysis	+
Citrate use	
Koser	+
Christensen	+
Simmons	+
$(NH_4)_2SO_4$ use	-
Pigment formation	
King A	-
KingB	-
Urease	+
Growth temperature	30
pH	6-9
Litmus milk	-

Table 1-1d. Taxonomical characteristics.

	71D
Dihydroxyaceton formation	-
Cellose hydrolysis	-
Malonic acid utilization	-
5% NaCl	-
DNase	-
Tween 80 hydrolysis	-
Vitamin requisistion	-
Gelatin liquefaction	-
n-hexadecan use	+

Table 1-2. Utilization of carbon sources by *Rrhodococcus opacus* and *R. wratislaviensis.*

Carbon source utilization was detected by means of reduction of MTT, enzymatic activity by cleavage of chromogenic substrates.

Carbon source	*R. opacus* [a,b]	*R. opacus* DSM 43205^{T} [b, d]	*R. opacus* 71D [c]	*R. wratislaviensis* N805^{T} [d]
	Positive (%)			
Acetamide	0	-	-	-
4-Aminobutyrate	60	+	+	-
Caprate	100	+	+	-
D-Galactose	100	+	+	-
Glutarate	0	-	-	-
2-Oxoglutarate	100	+	+	-
Phenylacetate	40	+	+	-
L-Rhamnose	0	-	-	+
D-Ribose	100	+	+	-
D-Arabitol	100	+	N.T.	-
L-Serine	60	-	+	-
L-Proline	20	-	+	+
L-Alanine	100	+	+	+
L-Asparate	100	+	+	+
Tyramine	100	+	+	-
Benzoate	60	+	-	+
o-Hydroxybenzoate	60	+	-	+
p-Hydroxybenzoate	100	+	+	+

(a) Inclusion: DSM 43204, DSM 43206, DSM 43250, DSM 43251

(b) These data were taken from S. Klatte *et al*. (1994). [66]

(c) This work

(d) These data were taken from M. Goodfellow *et al*. (2002). [67]

N.T; Not tested

Purification of NHase.

The results of purification of NHase from *R. opacus* 71D are summarized in Table 1-3. The enzyme was purified 256-fold with a yield of 12.3% from the cell-free extract. The specific activity of the purified enzyme was 1040 U mg^{-1} protein. The subunit M_r was estimated to be 32,300 (β-subunit) and 27,200 (α-subunit), respectively, by comparing mobilities on SDS-PAGE to that of standard proteins. Results of SDS-PAGE analysis of samples from the final step of the purification are shown in Figure 1-2. The molecular weight of purified native NHase was estimated to be about 119,000 from its mobility relative to standard proteins on gel filtration by HPLC.

Table 1-3. Summary of purification of NHase from *R.opacus* 71D.

Purification step	Total protein (mg)	Total activity[(a)] (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	1,840	7,500	4.07	100
$(NH_4)_2SO_4$ fractionation	1,170	6,200	5.28	82.6
Butyl -Toyopearl	131	5,020	38.3	66.9
DEAE-Toyopearl	25.1	3,510	140	46.8
Gigapite	16.7	3,490	209	46.6
Mono Q 5/5	2.29	2,140	934	28.5
Hydroxyapatite	0.88	919	1,040	12.3

(a) α-Aminobutyronitrile was used as a substrate.

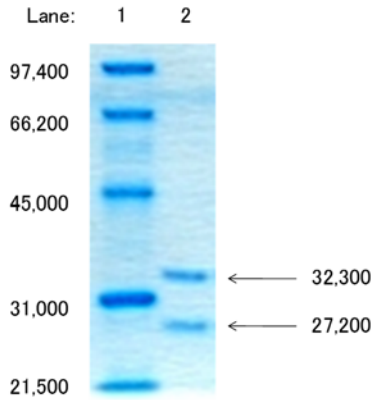


Figure 1-2. SDS-PAGE of purified NHase.

Lane 1: Molecular weight standards, Lane 2: Purified NHase. The protein standards used were phosphorylase b (M_r 97,400), bovine serum albumin (M_r 66,200), ovalbmin (M_r 45,000), carbonic anhydrase (M_r 31,000), and soybean trypsin inhibitor (M_r 21,500) for SDS-PAGE.

Effect of temperature and pH on NHase activity.

The optimal temperature for NHase activity was assayed at various temperatures for 5 min (Figure 1-3(A)). The reaction mixture (total volume 1.0 ml) was composed of 50 mM KPB (pH 7.0), 20 mM (*RS*)-α-ABN, 0.1 mM $CoCl_2$, and purified NHase (0.7 U). The optimal temperature of the NHase of *R. opacus* 71D was 20°C at pH 7.0. The stability of the enzyme was examined at various temperatures. After the enzyme (0.7 U) had been pre-incubated for 30 min in 20 mM KPB (pH 7.0), a sample of the enzyme

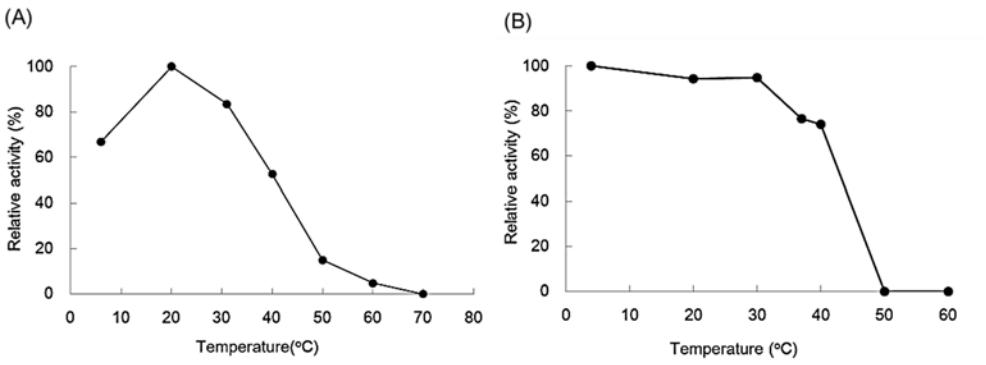


Figure 1-3. Optimum temperature (A) and temperature stability (B) for the activity of NHase from *R. opacus* 71D.

solution was taken, and the activity was assayed as described in the Experimental section. It exhibited the following levels of activity: 50°C, 0%; 40°C, 74%; 37°C, 77%; 30°C, 95%; 20°C, 94%; 4°C, 100% (Figure 1-3 (B)).

The optimal pH and pH stability for the activity were measured using the following buffers (final concentration 100 mM): Glycine-HCl (pH 2.0-4.0), acetic acid/sodium acetate (pH 3.0-6.0), KPB (pH 6.0-8.0), Tris-HCl (pH 8.0-9.0), Glycine-NaOH (pH 9.0-13.0), and McIlvaine (pH 3.0-8.0). After the enzyme (3.0 U) had been pre-incubated for 30 min at 30°C in 100 mM of each buffer, a sample of the enzyme solution was taken, and the activity was assayed as described in the Experimental section. The optimal pH of the enzyme was pH 8.0 (KPB) at 30°C and the enzyme was stable between pH 6.0 and pH 7.0 at 30°C for 30 min (Figure 1-4).

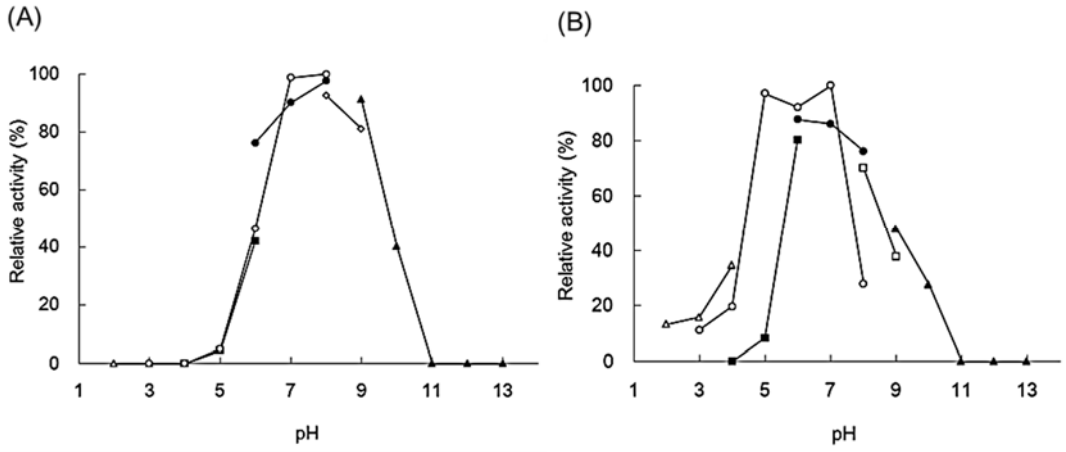


Figure 1-4. The optimal pH (A) and pH stability (B) for the activity of NHase from *R. opacus* 71D.

Inhibitors.

The purified enzyme solution (3.0 U) was treated with each 1 mM compound for 30 min at 30°C. The concentration of compounds was 1 mM in the reaction mixture. Residual activities were measured after the treatment following the assay method

described above.

The enzyme was strongly inhibited by thiol reagents, Ag^{2+}, Cu^{2+}, Hg^{2+}, and *p*-chloromercuribenzoate showing residual activity as 6.59, 30.9, 0.847, and 23.8%, respectively, while iodoacetate (105% residual activity) showed no significant inhibition of the activity of the enzyme. As carbonyl reagents, phenylhydrazine (62.9%) and PMS (79.8%) inhibited the enzyme, but D, L-penicillamine (147%) and D-cycloserine (126%) did not inhibit this enzyme. Chelating reagents such as EDTA (46.8%), EGTA (84.7%), and NaN_3 (86.6%) inhibited the activity of the enzyme. Ammonium peroxide (120%) (oxidizing reagent) and 2-mercaptoethanol (94.6%) (reducing reagent) had no effect on this enzyme but dithiothreitol (74.4%) (reducing reagent) inhibited the activity of the enzyme.

Substrate specificity and enantiomeric ratio.

Enzyme activities toward various nitriles were examined at a concentration of 20 mM (Table 1-4). The activity for α-ABN corresponding to 1040 U/mg was taken as 100%. The enzyme acted on a broad range of nitriles including acrylonitrile, *n*-butyronitrile, benzonitrile, alaninonitrile, phenylglycinonitrile, and mandelonitrile. Kinetic parameters for several racemic α-aminonitriles and methacrylonitrile with high affinities of known NHases such as *P. chlororaphis* B23[37], *Arthrobacter* sp. strain J-1[25], and *R. rhodochrous* J-1[40] were calculated by the Hanes-Woolf plot. The lowest K_m and highest V_{max} values were for valinonitrile and α-ABN, respectively, for the α-aminonitriles. NHase preferentially hydrated the *S*-enantiomer of α-aminonitriles rather than the *R*-enantiomer. However, as shown in Table 1-2, *E*-values[65] for the hydrolysis of α-aminonitriles were indicated to be 1.0-2.1 (*S*); therefore, this NHase was

characterized to catalyze non-stereoselective hydrolysis of α-aminonitriles.

Table 1-4. Substrate specificity and stereoselectivity of NHase from *R. opacus* 71D.

Substrate (20 mM)	Relative activity (%)	K_m (mM)	*E* value
Acrylonitrile[a]	146	N.D.	-
Propionitrile[a]	192	N.D.	-
n-Butyronitrile[a]	363	N.D.	-
n-Valeronitrile[a]	261	N.D.	-
Methacrylonitrile[b]	131	0.233	-
Isobutyronitrile[a]	56.1	N.D.	-
Benzonitrile[c]	72.9	N.D.	-
Mandelonitrile[c]	70.7	N.D.	-
Alaninonitrile	74.0	17.3	1.16 (*S*)
α-Aminobutyronitrile	100 (1040 U/mg)	4.33	1.22 (*S*)
Valinonitrile	15.1	0.200	1.05 (*S*)
Leucinonitrile	8.51	1.15	2.11 (*S*)
tert-Leucinonitrile	1.80	1.59	N.D.
Phenylglycinonitrile	29.1	3.63	1.76 (*S*)
Phenylalaninonitrile	42.9	1.58	1.00 (*S*)

The activity for α-aminobutyronitrile correspondindng to 1040 Umg^{-1} was taken as 100%.

[a] *Assay with aliphatic nitrile as a substrate* - The reaction mixture (total volume 1.0 mL) was composed of 50 mM KPB (pH 7.0), 20 mM aliphatic nitrile, and the appropriate amount of the enzyme. The enzyme was added to initiate the reaction. After incubation at 30°C, sample (0.1 ml) was taken from reaction mixture at 5 min. The reaction was stopped by adding 900 μl of ethylacetate. The amount of amide formed in the reaction mixture was determined with GC apparatus equipped with a β-Dex-325 column (Supelco, USA) using He as a carrier gas (Detector temperature: 230°C and injection temperature 220°C).

[b] The K_m value for methacrylonitrile was assayed at 30°C as the rate of the formation of methacrylamide from several concentration methacrylonitrile in 50 mM KPB (pH 7.0) containing 0.1 mM $CoCl_2$ by measuring absorbance at 224 nm ($\Delta\varepsilon = 3400\ M^{-1}cm^{-1}$).

[c] When benzonitrile and mandelonitrile were used as substrate, the amount of corresponding amides formed was determined by HPLC with MIGHTYSIL PR-18 GP column using the solvent system of 80% acetonitrile containing 10 mM H_3PO_4 at a flow rate of 1.0 ml min^{-1}.

Dynamic kinetic resolution of racemic α-aminonitrile to form chiral α-amino acid.

The time course of (*R*)-α-(ABA) conversion from racemic (*RS*)-α-ABN sulfuric acid salt by purified NHase, D-aminopeptidase (DAP) from *O. anthropi* C1-38[16] and ACL racemase is shown in Figure 1-5.

In the initial phase of the reaction, the NHase rapidly converted ABN to racemic α-ABA-NH_2 with *E*-value of 1.2. After 20 min, α-ABN was completely converted to (*R*)- and (*S*)-α-ABA-NH_2. (*R*)-ABA-NH_2 was hydrolyzed by DAP, while the remaining (*S*)-α-ABA-NH_2 was racemized by ACL racemase. The concentration of (*R*)-α-ABA was the same as that of α-ABN originally present in the reaction mixture. All of (*RS*)-α-ABN was converted to (*R*)-α-ABA in 6 h (conversion >99% with >99% ee). (*S*)-α-ABA also could be obtained by DKR of racemic ABN by purified NHase, L-amino acid amidase ($LaaA_{Bd}$) from *B. diminuta* TPU 5720[26] and ACL racemase (Figure 1-6). All of (*RS*)-α-ABN was converted to (*S*)- α-ABA in 12 h (conversion >99% with >99% ee).

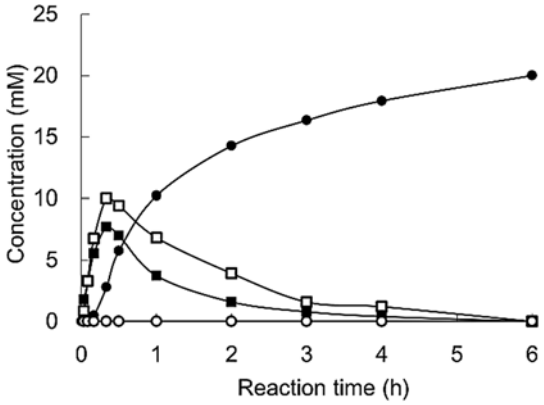


Figure 1-5. The enzymatic conversion of (*RS*)-α-aminobutyronitrile to (*R*)-α-aminobutyric acid.

The reaction mixture (total volume 1.0 ml) was composed of 50 mM KPB, pH7.0, 20 mM (*RS*)-α-aminobutyronitrile, 500 nM pyridoxal phosphate, NHase (1.0 U), DAP (0.52 U), and ACL racemase (1.6 U) at 30°C. The NHase was added to initiate the reaction.

Symbols: (*R*)-α-aminobutyramide (■), (*S*)-α-aminobutyramide (□), (*R*)-α-aminobutyric acid (●), and (*S*)-α-aminobutyric acid (○).

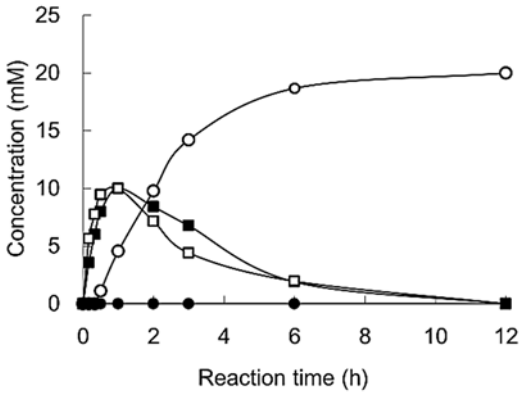


Figure 1-6. The enzymatic conversion to (*S*)-α-aminobutyric acid from (*RS*)-α-aminobutyronitrile.

The reaction mixture (total volume 1.0 ml) was composed of 50 mM KPB, pH7.0, 20 mM (*RS*)-α-aminobutyronitrile, 500 nM pyridoxal phosphate, the NHase (0.92U), $LaaA_{Bd}$ (0.39 U), and ACL racemase (1.6 U) at 30 °C. The NHase was added to initiate the reaction.

Symbols: (*R*)-α-aminobutyramide (■), (*S*)-α-aminobutyramide (□), (*R*)-α-aminobutyric acid (●), and (*S*)-α-aminobutyric acid (○)

Discussion

An α-aminopropionitrile hydrolyzing microorganism has been isolated using the substrate as a nitrogen source.[8] Considering that α-aminonitrile would be spontaneously decomposed to toxic aldehyde and cyanide ions, the author attempted the first screening for α-aminonitrile hydrolyzing microorganisms using *n*-butyronitrile as a sole carbon and nitrogen source because of its structural similarity to α-ABN and less toxic nature. In the second screening, the author selected a bacterium hydrolyzing α-ABN with lower stereoselectivity. Finally, the author isolated and identified *R. opacus* 71D exhibiting very low stereoselectivity toward α-ABN. It is capable of growing in a medium containing *n*-butyronitrile as a sole source of carbon and nitrogen and its NHase activity was induced by *n*-butyronitrile, as well as by various nitrile and amide compounds. Among the inducers tested, isovaleramide was selected as the best for enzyme formation, but it could not grow on nitriles as a sole carbon and nitrogen source such as methacrylonitrile, isobutyronitrile, isovarelonitrile, or aromatic nitriles such as benzonitrile and phenylacetonitrile, or α-aminonitriles such as (*RS*)-α-ABN and (*RS*)-α-ABN.

NHase from *R. opacus* 71D was purified 256-fold with a yield of 12.3% from the cell-free extract. The properties of this enzyme are summarized in Table 1-5.

The specific activity of the purified enzyme is 1040 U mg^{-1} protein for α-ABN as a substrate. From results of SDS-PAGE and gel filtration by HPLC, the molecular weight of this enzyme is about 119,000, forming an $\alpha_2\beta_2$ hetero-tetramer composed of α and β-subunits of NHase with molecular weights of 32,300 (β-subunit) and 27,200 (α-subunit), respectively. The purified enzyme was unstable when incubated in aqueous

Table 1-5. Characteristic of NHase from *R. opacus* 71D.

	Rhodococcus opacus 71D
Native molecular weight	119,000
Subunit	
(α)	27,200
(β)	32,300
Subunit structure	$\alpha_2\beta_2$
Prosthetic group	Co^{2+}
Optimum pH	8
Optimum Temperature	20°C
pH stability	6-8
Temperature stability	4-30°C
Inhibitor	$CuSO_4$, $AgNO_3$, $HgCl_2$, PCMB, 1 mM KCN (65%)

solution at temperatures higher than 30°C for 30 min (50% activity remained when it was incubated at 40°C for 30 min) and was labile to dilution. This enzyme was stabilized by the addition of 4 mM butyrate, as it has been reported that NHase can be stabilized by organic acids such butyrate or valerate.[15-17] The optimal pH, temperature, stability, and inhibitors are similar to other NHase from mesophilic bacteria such as *R. rhodochrous* J-1 (low-molecular mass NHase), *Rhodococcus* sp. N-774, and *P. chlororaphis* B-23, except for the KCN inhibition. NHase can be classified according to the presence of a metal cofactor such as ferric NHase or cobalt NHase. Generally, ferric NHases such as from *P. chrorolaphis* B23[37] and *Brevibacterium* sp. R312[68] are only slightly inhibited by KCN, whereas cobalt NHase such as that of *R. rhodochrous* J-1[40] enzyme is strongly inhibited by 0.01 mM KCN (Relative activity 38%). NHase from *R. opacus* 71D is not as sensitive to KCN. When incubated at 30°C for 30 min in the standard reaction mixture containing KCN at 1 mM, 5 mM, and 10 mM, the residual activity of this enzyme was 65%, 38%, and 20%, respectively. Thus, this enzyme is very

suitable for converting α-aminonitriles to corresponding amides because α-aminonitriles are usually unstable in aqueous solution and produce cyanide by the spontaneous retro-Strecker reaction.

NHase contained cobalt ions and also had wide substrate specificity, acting on aliphatic nitriles such as acrylonitrile, *n*-butyronitrile, α-ABN, and aromatic nitriles such as benzonitrile, mandelonitrile, and phenylglycinonitrile. Moreover, the author reported K_m and *E* values for several α-aminonitriles using the purified enzyme for the first time. This is the first report showing the substrate specificity of nitrile hydratase acting on α-aminonitriles and aliphatic and aromatic nitriles.

Recently, stereoselective NHase has been attracting attention since it had been believed to be not very stereoselective. Some NHase can hydrate (*RS*)-2-(4-isobutyl phenyl)-2-methylpropionitrile with *R* or *S*-stereoselectivity and *Rhodococcus* sp. strain HT 40-6 act on the *S*-enantiomer of mandelonitrile.[69] However, a stereoselective NHase acting on α-aminonitrile has been never reported. The author thus attempted to utilize a non-stereoselective NHase toward α-aminonitrile in DKR.

Asano et al. have reported the discoveries and properties of *R*-stereoselective amino acid amidases such as DAP, D-amino acid amidase (DaaA)[27], and alkaline D-peptidase[31] and *S*-steroselective amino acidc amide hydrolase such as $LaaA_{Bd}$ and L-amino acid amidase ($LaaA_{Pa}$) from *P. azotoformans* IAM 1603.[29] By combination of non-stereoselective NHase, ACL racemase and *R*- or *S*-stereoselective amidase, it is possible to construct an efficient system to produce the corresponding (*R*)- or (*S*)-α-amino acids from racemic α-aminonitriles. Actually, (*S*)-alanine with yielding 99% and 99% ee was converted from 50 mM alaninonitrile using NHase (1.6 U), ACL racemase (0.5 U), and $LaaA_{Pa}$ (2 U) in 3 hour. Moreover, using NHase, ACL racemase, and

$LaaA_{Bd}$, 10 mM valinonitrile, 30 mM leucinonitrile, and 10 mM phenylalaninonitrile were converted to the corresponding *S*-enantiomer of α-amino acids with yield 74.5-99% (Table 1-6). $LaaA_{Bd}$ acted on not only (*S*)-alaninamide and (*S*)-phenylalaninamide, but also *R*-form alaninamide and slightly phenylalaninamide.[26] (*S*)-Phenylalanine was produced from phenylalaninonitrile with a little bit low enantiomeric excess. (*R*)-Amino acids also would be produced from several racemic α-aminonitriles. DAP acted on (*R*)-amino acid amides with small substituents, such as (*R*)-alaninamide and (*R*)-α-aminobutyramide. On the other hand, DaaA showed hydrolyzing activity toward bulky amino acid amides with (*R*)-leucinamide and (*R*)-phenylalaninamide. Alaninonitrile (50 mM), 30 mM leucinonitrile, and 20 mM valinonitrile were converted to the corresponding (*R*)-α-amino acids (Table 1-7). To the best of knowledge, this is the first report on the dynamic kinetic resolution of racemic α-aminonitriles to the corresponding not only (*R*)-α-amino acids but also (*S*)-α-amino acids. This new method of dynamic kinetic resolution has a possibility to be developed to the large scale production of optically active α-amino acids.

Table 1-6. Conversion of α-aminonitrile to (*S*)-α-amino acid by DKR.

Substrate	Concentration (mM)	Reaction time (h)	Yield (%)	Enantiomeric excess (% ee)
Alaninonitrile[a]	50	3	>99	>99
α-Aminobutyronitrile	50	3	>99	>99
Valinonitrile	10	6	99.0	>99
Leucinonitrile	30	9	>99	>99
Phenylalaninonitrile[b]	10	12	73.6	97.5

The reaction (1 mL) was carried out using 1.6 U NHase, 0.5 U ACL racemase, and 1.8 U $LaaA_{Bd}$ at 30°C.

[a] The reaction (1 mL) was carried out using 1.6 U NHase, 0.5 U ACL racemase, and 2 U $LaaA_{Pa}$.

[b] The reaction (1 mL) was carried out using 1.6 U NHase, 1.0 U ACL racemase, and 1 U $LaaA_{Bd}$.

The yield and enantiomeric excess were determined by HPLC.

Table 1-7. Conversion of α-aminonitrile to (*R*)-α-amino acid by DKR.

Substrate	Concentration (mM)	Reaction time (h)	Yield (%)	Enantiomeric excess (% ee)
Alaninonitrile[a]	50	3	>99	>99
α-Aminobutyronitrile[a]	50	3	>99	>99
Valinonitrile	20	20	>99	>99
Leucinonitrile	30	9	>99	>99

The reaction (1 mL) was carried out using 1.6 U NHase, 0.5 U ACL racemase, and 1.9 U DaaA at 30°C.

[a] The reaction (1 mL) was carried out using 1.6 U NHase, 0.5 U ACL racemase, and 1.8 U DAP.

The yield and enantiomeric excess were determined by HPLC.

Chapter II

Identification of nitrile hydratase gene and expression in *E. coli*

Previous chapter, the author succeeded in the demonstration of dynamic kinetic resolution of chiral α-amino acid from α-aminonitrile using three purified enzymes including nitrile hydratase (NHase) from *Rhodococcus opacus* 71D which was newly isolated, stereoselective amidase and α-amino-ε-caprolactam (ACL) racemase from *Achromobacter obae*. The author's way of constructing enzymatic process is in the order of (i) by purified enzyme and (ii) by cell-free synthesis. In the order of (i) to (iii), it becomes difficult to achieve high e.e. of the product, especially in this kind of cascade reactions.

In this chapter, enzymatic properties of NHase, a useful biocatalyst for converting to racemic α-amino acid amide from racemic α-aminonitrile, was characterized in detail. NHase gene from *R. opacus* 71D was expressed in *E. coli* JM109 under downstream region of *lac* promoter for efficient production process of chiral α-amino acid. Under optimum conditions, 200 mM of (*R*)-α-aminobutyric acid (yield >99% and >99% ee) was produced in 18 h using cell-free extract of three recombinants including NHase, D-aminopeptidase from *Ochrobactrum anthropi* C1-38, and ACL racemase.

Experimental section

Materials.

Escherichia coli JM109 (*e14*$^-$ (*mcr*A$^-$), *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, (r_K^- m_K^-), *sup*E44, *rel*A1, λ^-, Δ (lac-proAB), [F'*tra*D36, *pro*AB$^+$, *lac*I^q, lacZΔM15] was used as a host for DNA manipulation and expression. Plasmid pUC18 (Takara Bio, Ohtsu, Japan) was used as a vector for *E. coli*.

Cloning of the NHase gene from* R. opacus *71D.

The chromosomal DNA from *R. opacus* 71D was isolated by the method of Saito and Miura.[70] Oligonucleotide primers 5'-ATGAACGGCGTNTTCGATCTAGG-3' and 5'-TGTGTACGCAANYKCGTGTCGGT-3' were designed on the N-terminal amino acid sequences of α- and β-subunit of NHase. Reaction mixture (50 μl) for the PCR contained 25 μl 2 X GC buffer, 2.5 mM dNTP Mixture, 100 pmol each primers, 100 ng template, and 2.5 U *LA Taq*. The PCR product (0.7 kb) was cloned pT7blue vector in *E. coli* and nucleotide sequencing was performed using the dideoxynucleotide chain-termination method with M13 forward and reverse oligonucleotides as primers. Sequence reactions were carried out with a Thermosequenase cycle sequencing kit and dNTP mixture with DNA Thermo sequenase DNA polymerase with pyrophosphatase (Pharmacia Biotech, Tokyo, Japan). Inverse PCR was performed as described by Ochman et al.[71] The chromosomal DNA from *R. opacus* 71D was digested with *Fba*I, *Nco*I, *Eco*RI, or *Sca*I and circularized by ligation. Reaction mixture (50 μl) for the inverse PCR contained 25 μl 2 X GC buffer, 2.5 mM dNTP Mixture, 100 pmol each primers, 100 ng template, and 2.5 U *LA Taq*. The amplified PCR product was analysed

using ABI PRISM 310 Genetic analyzer. Homology search was performed with the sequence similarity searching programs BLAST and ClustalW method was used to align the sequence. The genetyx software system (Software Development Co., Tokyo, Japan) was used for computer analysis of nucleotide sequences and deduced amino acid sequences. The nucleotide sequences data of NHase in this study has been submitted to the GenBank/EMBL/DDBJ Data Bank with accession number AB481223.

Construction of the plasmid and expression of the NHase gene from R. opacus 71D in E. coli.

The gene for the α and β-subunits of NHase and p15K were amplified by PCR with the primer set 5'-GCCGGGATCCCaaggagTTTCGC**ATG**AACG-3' and 5'-TGCTCGAAGCTTAGTGTGGGTTAGCTGGTC-3' (restriction site, Shine-Dalgarno sequence, and initiation codons are shown with underlines, lowercase letters, and bold letters, respectively) using DNA of *R. opacus* 71D as a template. The amplified fragment was cloned between *Bam*HI and *Hin*dIII sites of pUC18 to produce pNH1.

NHase α and β-subunit genes and the p15K gene were amplified from pNH1 with the primer set 5'-GCCGGGATCCCaaggagTTTCGC**ATG**AACG-3' and 5'-TCCTGGTGTAGCGGTACCCTCATGAGGCTG-3' and 5'-GCCCGGTACCaaggagTGCCTC**ATG**AGCGCCTCGCTACACCAGGA- 3' and 5'-TGCTCGAAGCTTAGTGTGGGTTAGCTGGTC-3', respectively. After two PCR products were ligated at the *Kpn*I site, the ligated DNA fragment was inserted between *Bam*HI and *Hin*dIII sites of pUC18 and the plasmid pNH2 was obtained.

To express active NHase in *E. coli* JM 109, the author constructed NHase expression plasmids, and transformed *E. coli* JM109. Recombinant *E. coli* was grown in

LB medium containing ampicillin (80 μg/ml) at 37ºC for 7 h. Then, 1 mM IPTG and 1 mM $CoCl_2$ were added to the culture, and incubated at 30ºC for an additional 12 h.

Enzymatic synthesis of (R)-α-aminobutyric acid from (RS)-α-aminobutyronitrile.

The reaction mixture contained 1 mmol KPB, pH 8.0 (100 mM), 20 nmol PLP (2 μM), 1 μmol $CoCl_2$ (0.1 mM), the cell-free extract of *E. coli* pNH2 (6.4 U as NHase), *E. coli* pDAP1 (19.9 U as DAP), the heat treated cell-free extract of *E. coli* pACL60 (1.4 U as ACL racemase), and 0.266 g (*RS*)-ABN (200 mM) in a total volume of 10 ml. After the reaction mixture was incubated at 30ºC for 18 h, it was adjusted to pH 1.0 with concentrated HCl, and then filtrated and neutralized with 6 N NaOH. The solution was evaporated *in vacuo* and recrystallized from water-methanol. Finally, (*R*)-α-ABA was obtained at a yield of 0.163 g (79.0%) with 99.9% ee as colorless crystals; $[\alpha]^{24}_{D}$ -19.6 º (*c* 1.00, H_2O).

Results

***Production of NHase in* E. coli.**

When the *E. coli* JM109 transformant harboring pNH1 was cultivated in LB medium supplemented with ampicillin, IPTG, and $CoCl_2$ for 24 h at 37°C, NHase activity was not detected in the cell-free extract solution of the transformant, while activity was shown when the induction temperature was set to less than 30°C. *E. coli* JM109/pNH1 showed NHase activity (2,450 U/g acetone dried cells) toward substrates such as α-ABN. Activity was 7 times higher per mg dry cells than that of *R. opacus* 71D (336 U/g acetone dried cells). Thus, the author improved the expression of NHase by replacing the overlapped sequence between the α-subunit stop codon and p15K start codon, with a new SD sequence (AAGGAG) and start codon, and constructed the pNH2 plasmid (Figure 2-1). After *E. coli* JM109 harboring pNH2 was cultivated in LB medium supplemented with ampicillin for 7 h at 37°C, 1 mM IPTG and 1 mM $CoCl_2$ were added to the culture, which was then incubated aerobically at 30°C for an additional 12 h. Because NHase activity per mg dry weight cells of *E. coli* harboring pNH2 (11,000 U/ g acetone dried cells) was about 30 times higher than that of *R. opacus* 71D, the author decided to use the much improved plasmid pNH2 for NHase production.

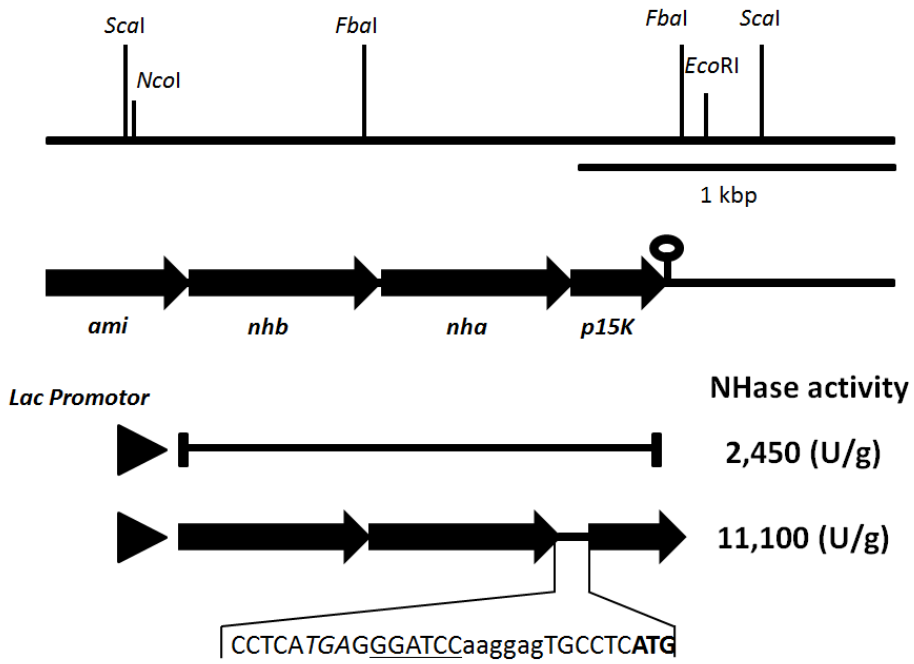


Figure 2-1. Production of NHase from *R. opacus* 71D in recombinant *E. coli*.

Restriction site, Shine-Dalgarno sequence, initiation codon of p15K, and stop codon of α-subunit are shown in underline, lowercase, bold letters, and italic letters respectively.

Enzymatic synthesis of (R)-α-aminobutyric acid from (RS)-α-aminobutyronitrile.

The optimum temperature was similarly assayed by measuring the product formed at different temperatures with 100 mM KPB; pH 8.0, 2 μM PLP, 50 mM (*RS*)-α-ABN, 0.1 mM $CoCl_2$, purified NHase, ACL racemase, and DAP (Figure 2-2 (A)). Optimal pH for the conversion was measured using the following buffers: KPB (pH 6.0-8.0) and Tris-HCl (pH 7.0-9.0). The reaction mixture (total volume 1.0 ml) was composed of 100 mM buffer, 2 μM PLP, 50 mM (*RS*)-α-ABN, 0.1 mM $CoCl_2$, purified NHase, ACL racemase, and DAP (Figure 2-2(B)). From these results, optimum conditions for the production of (*R*)-α-ABA were determined to be a pH 8.0 at 30°C.

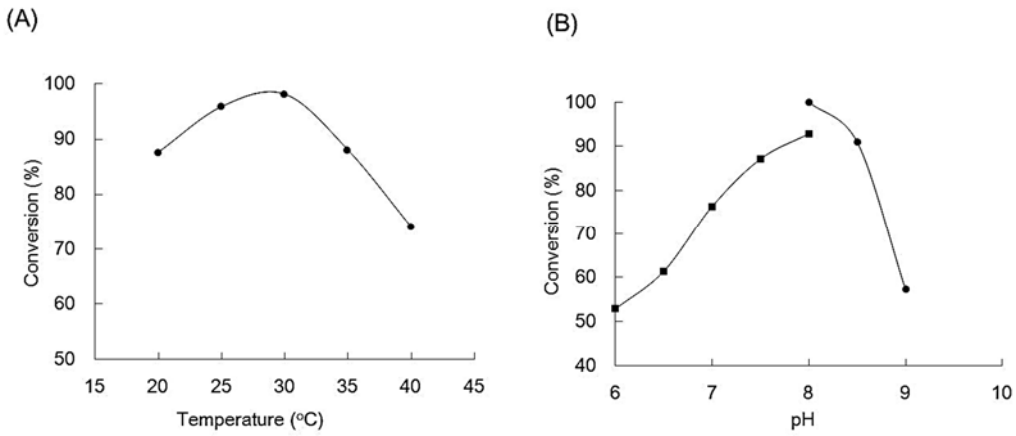


Figure 2-2. Optimum temperature (A) and pH (B) for conversion of (*RS*)-α-aminobutyronitrile to (*R*)-α-aminobutyric acid.

(B) Symbol: (■), KPB (pH 6.0-8.0) and (○), Tris-HCl (pH 7.0-9.0).

Figure 2-3 shows the typical time course of DKR of α-ABN under optimum conditions (pH 8.0 at 30°C). α-ABN was added to the reaction mixture intermittently with a portion of 0.133 g (1 mmol) twice because NHase and ACL racemase were strongly inhibited by higher concentrations of the substrate. The first portion of the substrate was quickly converted to racemic α-ABA-NH_2 in less than 10 min. The resulting racemic α-ABA-NH_2 (100 mM) was quickly hydrolyzed by DAP to form (*R*)-α-ABA. Moreover, the remaining *S*-enantiomer was racemized by ACL racemase. After the 30 min reaction, another portion of the 1 mmol substrate was added to the reaction mixture. Racemic α-ABN (200 mM) was completely converted to (*R*)-α-ABA (200 mM) with a 99% yield and 99% ee after an 18 h reaction.

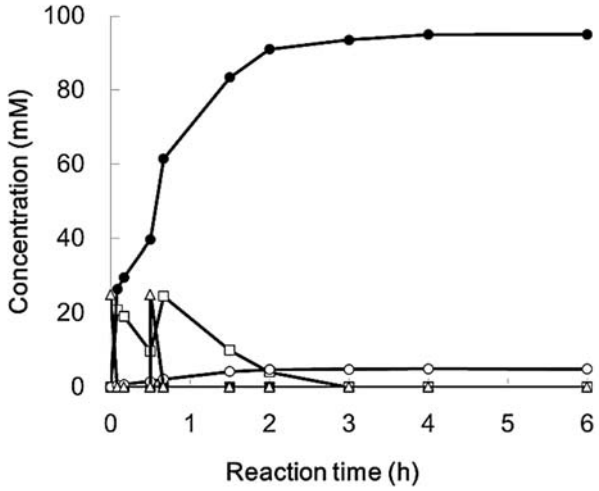


Figure 2-3. DKR of α-aminobutyronitrile to form (*R*)-α-aminobutyric acid using NHase, D-aminopeptidase and ACL racemase.

Symbols: (*R*)-α-aminobutyramide (■), (*S*)-α-aminobutyramide (□), (*R*)-α-aminobutyric acid (●), and (*S*)-α-aminobutyric acid (○).

Discussion

The author cloned the NHase gene from *R. opacus* 71D and determined its sequence and flanking region. The amidase gene was found to be located upstream of the β-subunit gene, and surprisingly, these genes overlapped with each other at the stop codon and start codon (ATGA sequence). Most amidase genes were found approximately 100 bp upstream of NHase ORF, except in *R. rhodochrous* J-1, with an amidase gene being located 1.9 kbp downstream of the α-subunit of the low molecular weight NHase gene.

Moreover, the author amplified the aldoxime dehydratase (Oxd) gene from *R. opacus* 71D by the method as described by Kato et al.[72] OxdB4-S3/OxdB4-AS2 primer pairs allowed the amplification of a 450 bp DNA fragment from this strain and protein

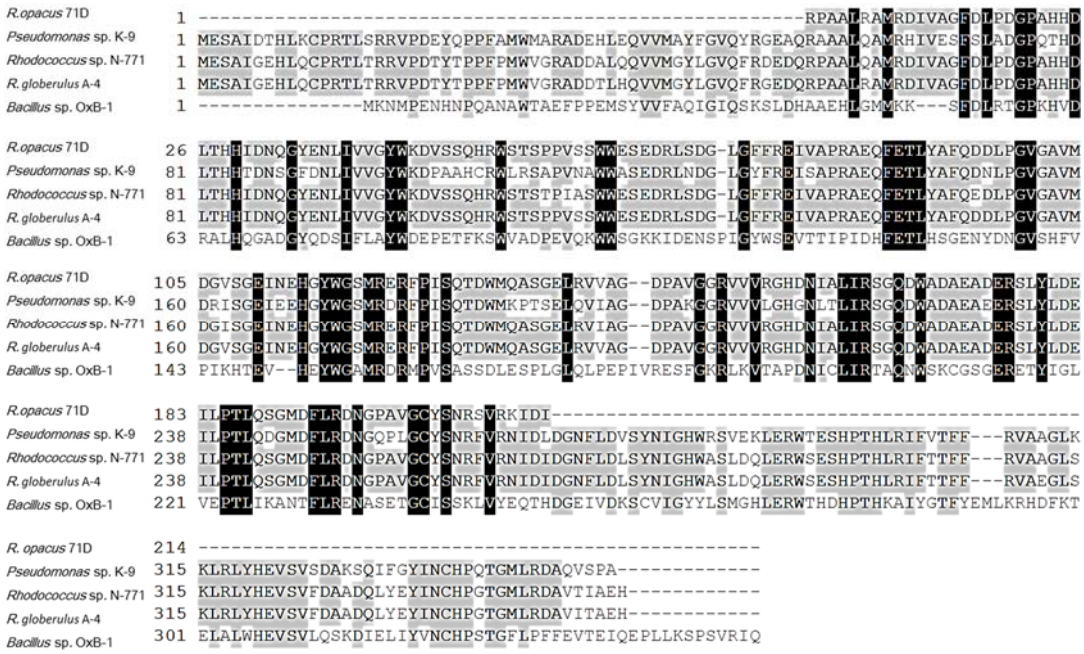


Figure 2-4. Alignment of amino acid sequences of Oxd from *R. opacus* 71D, *Pseudomonas* sp. K-9, *Rhodococcus* sp. N-771, *R. globerulus* A-4, and *Bacillus* sp. OxB-1.

Identical amino acids are marked in black and similar amino acids are marked in gray. The sequences have the following accession numbers: Oxd from *Pseudomonas* sp. K-9, AB193508; *Rhodococcus* sp. N-771, AB094201; *R. globerulus* A-4, AB105912; and *Bacillus* sp. OxB-1, AB028892.

encoded by this fragment showed similarities to the known Oxd from *Pseudomonas* sp. K-9, *R. erythropolis* N-771, and *R. globerulus* A-4 (Figure 2-4). An inverted repeat sequence was also found downstream of the p15K gene. Based on these results, *R. opacus* 71D was shown to have an aldoxime-nitrile pathway gene cluster.

Alignment of the amino acid sequence showed that the deduced primary structure of the α-subunit was similar to known cobalt containing NHase such as those from *Pseudonocardia thermophila* (identity: 64.1%), *Rhodococcus* sp. Cr4 (60.5%), *R. rhodochrous* J-1 (60.1%), *Bacillus* sp. RAPc8 (55.6%), and *Bacillus* sp. BR449 (55.2%). Analysis of the amino acid sequence of the β-subunit showed weak identities and significant homologies to *P. thermophila* (identity: 57.1%), *Rhodococcus* sp. Cr4 (45.3%), *R. rhodochrous* J-1 (45.3%), *Bacillus* sp. RAPc8 (38.8%), and *Bacillus* sp.

BR449 (38.8%). The α-subunit of NHase revealed that it contained a characteristic cobalt-binding motif, CSLCSC[73], which differed from the proposed iron-binding motif of other NHases (Figure 2-5).

It has been reported that the downstream region (activator protein) of the NHase gene is necessary to functionally express NHase in *E. coli*.[74] In the author's initial experiment, the expression level of NHase was very low when NHase and p15K genes were placed under the *lac* promoter in the pUC18 vector. Accordingly, the author replaced an overlapped sequence found between the α-subunit stop codon and the p15K start codon, with a new SD sequence and a start codon. NHase productivity per mg dry *E. coli* cells weight was elevated to about 30 times more than *R. opacus* 71D.

In chapter I, the author reported the one-pot synthesis of chiral α-amino acids by DKR of α-aminonitriles using purified three enzymes. In this study, non-natural (*R*)-α-aminobutyric acid was synthesized at a high concentration from racemic α-aminobutyronitrile using cell-free extracts of *E. coli* pNH2, *E. coli* pACL60, and *E .coli* pDAP1. Previous chapter is demonstration of the same reactions catalyzed by three purified enzymes. Cell-free synthesis, especially in this kind of cascade reactions is much more difficult to achieve high ee of the product because there is a risk of side reactions catalyzed by other enzymes originated from *E. coli*, such as L-amino acid amide hydrolase, amino acid racemase, and enzymes catalyzing decomposition of amino acid and amino acid amide. In this study, non-natural (*R*)-α-ABA was synthesized at a high concentration from racemic α-ABN using cell-free extracts of *E. coli* pNH2, *E. coli* pACL60, and *E .coli* pDAP1. DKR of (*RS*)-α-ABN (100 mM) to form (*R*)-α-ABA was carried out at optimum conditions using cell-free extracts of *E. coli* pNH2, *E. coli* pACL60, and *E. coli* pDap1. However, the optical purity of the (*R*)-

α-ABA synthesized showed only 68.6% ee because of the existence of a natural *S*-amidase in *E. coli*. Since ACL racemase is very stable up to 60°C for 10 min, the cell-free extract of *E. coli* pACL60 was treated at 60°C for 10 min and successfully used for DKR of α-ABN to inactivate *S*-amidase. When the concentration of α-ABN was high, α-ABA-NH_2 was obtained in lower yields such as 100, 100, 26.8, and 9.2% yields from 50, 100, 150, and 200 mM α-ABN, respectively, in 2 h. α-Aminonitrile was used at a low concentration (1 mM) because NHase was sensitive to even low concentrations. [46] On the other hand, NHase activity in this study was not affected much up to 100 mM α-ABN and substrate sharply decreased when 100 mM substrate was used. ACL racemase was also inhibited in the presence of 10 mM α-ABN (relative activity; 21.3%) and was completely inhibited at 50 mM. Therefore, the substrate needed to be quickly and completely converted to the product. Finally, the author successfully carried out fed-batch reactions to produce (*R*)-α-ABA at a yield of 99% and 99% ee with cell-free extracts of *E. coli* pNH2 and *E. coli* pDap1 and a heat-treated cell-free extract of *E. coli* pACL60.

(A)

(B)

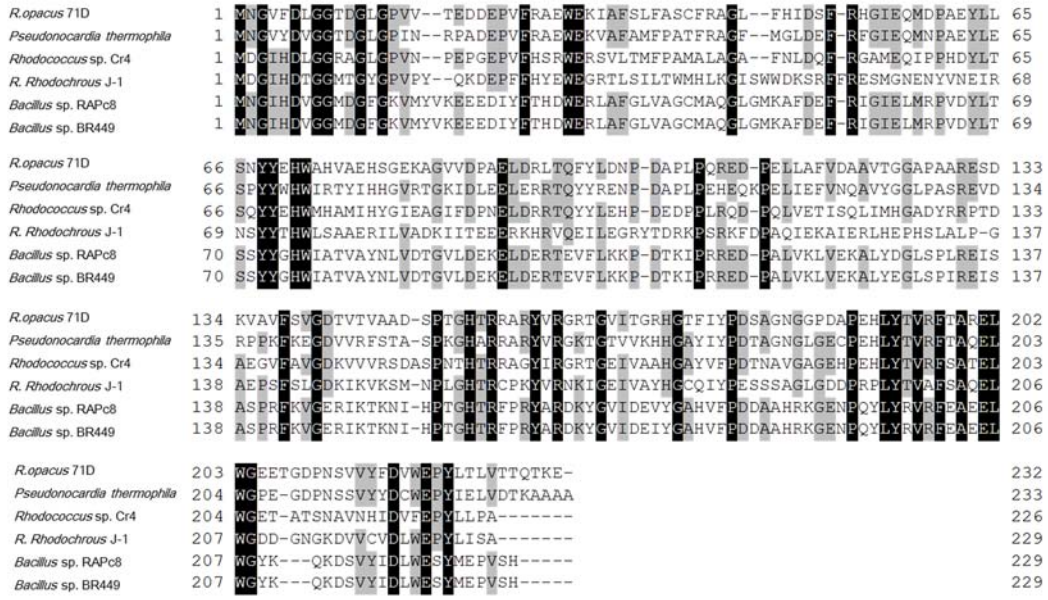


Figure 2-5. Alignment of amino acid sequences of α-subunit (A) and β-subunit (B) of NHase from *R. opacus* 71D, *P. thermophila*, *Rhodococcus* sp. Cr4, *R. rhodochrous* J-1, *Bacillus* sp. RAPc8, and *Bacillus* sp. BR449.

Identical amino acids are marked in black and similar amino acids are marked in gray. The cobalt binding site is marked with the red box. The sequences have the following accession numbers: NHase from *R. opacus* 71D, AB481223; *P. thermophila*, Q7SID2 (α), Q7SID3 (β); *Rhodococcus* sp. Cr4, AX538021 (α), AX538019 (β); *R. rhodochrous* J-1, P21219 (α), P21220 (β); *Bacillus* sp. RAPc8, AF257489 (α), AF257488 (β); and *Bacillus* sp. BR449, AF257489 (α), AF257488 (β).

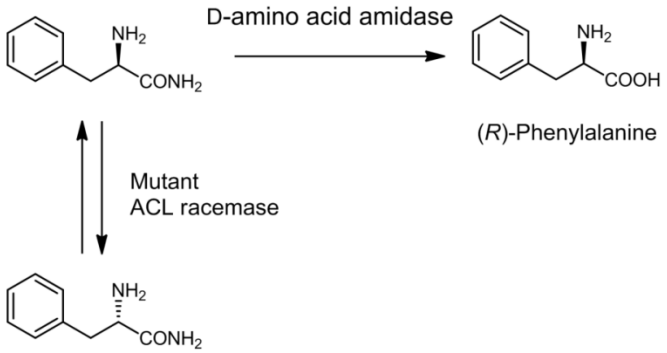
Chapter III

Enzymatic synthesis of chiral phenylalanine derivatives by a dynamic kinetic resolution of corresponding amide and nitrile substrates with multi enzyme system

In the previous chapter, the author succeeded in production of chiral α-amino acids such as *R* and *S* isomers of aliphatic amino acids by dynamic kinetic resolution (DKR) of the corresponding racemic α-aminonitrile using *R*- or *S*-stereoselective amidase and α-amino-ε-caprolactam (ACL) racemase.[75] However, ACL racemase has narrow substrate specificity, preferentially acting on small α-amino acid amides such as alaninamide, α-aminobutyramide and leucinamide, but hardly racemizes α-amino acid amides with bulky side chains such as phenylalaninamide.

Recently, directed evolution has become one of the methods for improving biocatalysts without detailed knowledge of enzyme structure and catalytic mechanism.[76] In earlier study, our laboratory obtained a mutant ACL racemase (L19V/L78T) with high phenylalaninamide racemizing activity by directed evolution based on the crystal structure of natural enzyme.[77]

In this chapter, the author describes an efficient synthetic method for (*R*)-phenylalanine and (*R*)-phenylalanine derivatives by DKR of corresponding amide and nitrile substrates with multi enzyme system (Scheme 3-1).



Scheme 3-1. Dynamic kinetic resolution of (*RS*)-phenylalaninamide to form (*R*)-phenylalanine.

Experimental section

Materials.

Restriction enzymes, DNA modifying enzymes, pUC18, and pSTV29 vectors were from Takara Shuzo Co., Ltd. (Kyoto, Japan). All other chemicals were from commercial sources.

Analytical methods.

Optical rotations were measured on a SEPA-300 (Horiba, Ltd., Kyoto, Japan). ^{1}H NMR spectrum was recorded on Bruker Biospin AVANCE II 400 (Bruker Biospin, Rheinstetten, Germany) system. The reaction products were characterized by MS using a Bruker-Daltonics micrOTOF instrument with an ESI source in positive mode (Bruker-Daltonics, Bremen, Germany). Data evaluation was performed using the Generate Molecular Formula software suite within Bruker Daltonics micrOTOF DataAnalysis version 3.4. Concentration and enantiopurity of all α-amino acid amides and α-amino acids were determined by HPLC equipped with a Crown Pak CR (+) column (Daicel Chemical Industries, Ltd., Osaka, Japan) using a solvent system of 60 mM $HClO_4$/10%

methanol.

Construction of pACLmut and pDBFB40.

Plasmid pET15ACL[77] containing the gene coding for the ACL racemase was used as a template in mutagenesis. The author targeted the mutagenesis to residues that interact with the 4 angstrom in the ligand, ε-caprolactam of active site, based on the crystal structure.[77] These residues have been identified as L19, W49, L78, M293, T295 and W436. Mutation library was constructed to contain possible amino acid changes at these residues. This library was screened against (*R*)-phenylalaninamide as substrate. After screening, L78T, L78S, and L78V single mutants were having higher activity than the native enzyme. These mutants were used as the parent for the second round of mutagenesis and screening. As in the second screening, the mutagenesis was performed on residues within the 6 angstrom in the ligand. Mutant ACL racemase (L19V/L78T) having both of L19V and L78T mutations was found to have the higher activity than the parent. The details of gene mutation and the enzymatic properties of the mutant ACL racemase will be described elsewhere. Mutant ACL racemase gene was inserted downstream of the *lac* promoter in high-copy-number plasmid pUC18, yielding pACLmut.

D-amino acid amidase gene from *Ochrobactrum anthropi* SV3 was amplified with oligonucleotides primer 5'-GAAATAAGCTTTAaggaggAATAGCCG**ATG**AGTG-3' and 5'-TTGAAGGTACCCTAACTGCGGGAG-3' (restriction site, Shine-Dalgarno sequence, and initiation codon are shown in underline, lowercase and bold letters, respectively) from plasmid pDAA-BFB40[18], digested with *Hind*III and *Kpn*I and inserted into low-copy-number plasmid pSTV29 vector to give pDBFB40. Plasmid

pACLmut and pDBFB40 were introduced into *E. coli* JM109, and the resulting transformant (*E. coli* pACLmut/pDBFB40) was cultivated in LB medium supplemented with ampicillin (80 μg/ml), and chloramphenicol (30 μg/ml).

***Preparation of enzyme from recombinant* E. coli.**

Recombinant *E. coli* transformants harboring pACLmut and pDBFB40 was grown in LB medium containing ampicillin and chloramphenicol at 37ºC for 24 h. Cells were harvested by centrifugation at 9,500 x *g* for 5 min at 4°C, and washed with 0.85% NaCl. The washed cells from 2 L culture were resuspended in 20 mM potassium phosphate buffer (KPB) and disrupted by sonication for 20 min (19 kHz, Insonator model 201M; Kubota, Tokyo, Japan). Cell-free extract was prepared by centrifugation of the lysate at 15,000 x *g* for 20 min at 4°C.

Definition of ACL racemase activity.

Activity of ACL racemase was measured by detecting the formation of (*R*)-phenylalanine via (*R*)-phenylalaninamide from (*S*)-phenylalaninamide at 30ºC. The standard assay solution contained 100 mM KPB (pH 7.0), 2 μM PLP, and 20 mM (*S*)-phenylalaninamide. One unit of enzyme activity was defined as the amount of enzyme catalyzing the conversion of substrate to the product at a rate of 1 μmol/min.

Definition of D-amino acid amide amidase activity.

D-amino acid amide amidase (DaaA) activity was measured by the method of Komeda et al.[18] The standard reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 20 mM (*R*)-phenylalaninamide and appropriate amount of the enzyme. One unit of

enzyme activity was defined as the amount of enzyme catalyzing the conversion of substrate to the product at a rate of 1 μmol/min.

Preparation of α-amino acid amides.

Preparation of α-amino acid amides from aldehydes.

α-Aminonitriles were obtained by Strecker synthesis with 0.10 mol aldehyde, 0.12 mol sodium cyanide, and 0.15 mol ammonium chloride. The reaction products were extracted with ethylacetate, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Products were diluted with MeOH/MeCN (1/9) and sulfuric acid was dropped. After filtration, α-aminonitrile sulfuric acid salt was obtained. A reaction mixture containing α-aminonitrile sulfuric acid salt was added to acetone, H_2O and 5.0 eq NaOH was stirred at room temperature for 24 h. The acetone layer containing α-amino acid amide was obtained and then pH was adjusted to pH 7.0 by concentrated HCl. After stirring at room temperature for 3 h, reaction mixture was filtrated and α-amino acid amide HCl was obtained as white crystals. α-Amino acid amide HCl synthesized included (*RS*)-phenylglycinamide HCl, 4-chloro-(*RS*)-phenylglycinamide HCl, 4-methyl-(*RS*)-phenylglycinamide HCl, and (*RS*)-homophenylalaninamide HCl.

Preparation of α-amino acid amides from α-amino acids.

α-Amino acid amides were prepared from α-amino acids by the method as described by Asano et al.[16] Methylester α-amino acids were synthesized by dropping 2.5 eq of thionyl chloride into α-amino acids suspended anhydride MeOH at 0°C. After the reaction was finished, methanol and HCl gas were evaporated *in vacuo* and α-amino acid methylester HCl was obtained as white crystals. α-Amino acid amides were

synthesized by ammonolysis of amino acid methylesters in anhydride MeOH saturated with dry ammonia gas at 0°C. α-Amino acid amide HCl synthesized included (*RS*)-phenylalaninamide HCl, (*R*)-phenylalaninamide HCl, (*S*)-phenylalaninamide HCl, (*RS*)-tyrosinamide HCl, 4-fluoro-(*RS*)-phenylalaninamide HCl, 3-fluoro-(*RS*)-phenylalaninamide HCl, 2-fluoro-(*RS*)-phenylalaninamide HCl, 4-chloro-(*RS*)-phenylalaninamide HCl, 4-bromo-(*RS*)-phenylalaninamide HCl, and 4-fluoro-(*RS*)-phenylglycinamide HCl.

Enzymatic synthesis of (R)-phenylalanine from (RS)-phenylalaninamide.

The reaction mixture contained 1 mmol Tris-HCl buffer, pH 8.0 (100 mM), 20 nmol PLP (2 μM), the cell-free extract of *E. coli* pACLmut/pDBFB40 (11 U of mutant ACL racemase and 1300 U of DaaA) from 50 ml culture, and 0.8 g (*RS*)-phenylalaninamide HCl (400 mM) in a total volume of 10 ml. When the reaction mixture was incubated under stirring at 40°C for 22 h, it was adjusted to pH 1.0 using concentrated HCl and then, the solution was filtrated and neutralized with 6 N NaOH. The reaction mixture was evaporated *in vacuo* and recrystallized from water-methanol. Finally, (*R*)-Phenylalanine was obtained in yield of 0.553 g (84%) with 99% ee as colorless crystals; $[\alpha]^{24}_{D}$ 35.0° (*c* 1.00, H_2O). ^1H NMR (D_2O, 400 MHz) δ: 7.2-7.4 (m, 5H), 3.9 (dd, 1H, *J* = 7.9, 5.2 Hz), 3.2 (dd, 1H, *J* = 14.5, 5.2 Hz), 3.0 (dd, 1H, *J* = 14.5, 8.0 Hz). MS m/z: calcd for $C_9H_{12}N_1O_2$ $[M+H]^+$ 166.0863; found, 166.0884.

Enzymatic synthesis of (R)-tyrosine from (RS)-tyrosinamide.

The reaction mixture contained 0.5 mmol Tris-HCl buffer, pH 8.0 (100 mM), 10 nmol PLP (2 μM), the cell-free extract from 25 ml culture (5.3 U of mutant ACL

racemase and 650 U of DaaA), and 0.4 g of (*RS*)-tyrosinamide HCl (1.8 mmol) in a total volume of 5 ml. When the reaction mixture was incubated with stirring at 40ºC for 24 h, the reaction mixture was adjusted to pH 1.0 with concentrated HCl and then, aggregate proteins of the cell-extract were removed by vacuum filtration and the solution was neutralized with 6 N NaOH. The precipitated (*R*)-tyrosine was filtrated, washed with cold water, and (*R*)-tyrosine was obtained in yield of 0.247 g (74%) with >99% *ee* as colorless crystals; $[\alpha]^{24}_{D}$ 8.0º (*c* 1.00, 6.2 N HCl). ^{1}H-NMR (D_2O, 400 MHz) δ: 6.8-7.1 (m, 4H), 3.8 (dd, 1H, *J* = 7.8, 5.2 Hz), 3.2 (dd, 1H, *J* = 14.7, 5.1 Hz), 3.0 (dd, 1H, *J* = 14.7, 7.8 Hz). MS m/z: calcd for $C_9H_{12}N_1O_3$ $[M+H]^+$ 182.0812; found, 182.0853.

Enzymatic synthesis of 4-fluoro-(R)-phenylalanine from 4-fluoro-(RS)-phenylalaninamide.

The reaction mixture contained 0.5 mmol Tris-HCl buffer, pH 8.0 (100 mM), 10 nmol PLP (2 μM), the cell-free extract from 25 ml culture (5.3 U of mutant ACL racemase and 650 U of DaaA), and 0.44 g of 4-fluoro-(*RS*)-phenylalanineamide HCl (400 mM) in a total volume of 5 ml. When the reaction mixture was incubated with starting at 40ºC for 24 h, the reaction mixture was adjusted to pH 1.0 with concentrated HCl and then, the solution was filtrated and neutralized with 6 N NaOH. The reaction mixture was evaporated *in vacuo* and recrystallized from water-methanol., 4-fluoro-(*R*)-phenylalanine was obtained in yield of 0.270 g (73%) with 99% *ee* as colorless crystals; $[\alpha]^{24}_{D}$ 7.8º (*c* 1.00, H_2O). ^{1}H NMR (D_2O, 400 MHz) δ: 7.0-7.3 (m, 4H), 4.1 (dd, 1H, *J* = 7.5, 5.6), 3.2 (dd, 1H, *J* = 14.7, 5.5 Hz), 3.1 (dd, 1H, *J* = 14.7, 7.6 Hz). MS m/z: calcd for $C_9H_{11}F_1N_1O_2$ $[M+H]^+$ 184.0768; found, 184.0789.

Enzymatic synthesis of (S)-phenylalanine from (RS)-phenylalaninamide.

The reaction mixture contained 0.5 mmol KPB, pH 7.0 (100 mM), 500 μmol $CoCl_2$ (0.1 mM), 10 nmol PLP (2 μM), the cell-free extract from 25 ml culture (7.3 U of mutant ACL racemase and 46 U of $LaaA_{Bd}$), and 2.0 mmol (*RS*)-phenylalaninamide HCl (400 mM) in a total volume of 5 ml. After the same workup procedure as described in Experimental section "*Enzymatic synthesis of (*R*)-phenylalanine from (*RS*)-phenylalaninamide*" in Chapter III, (*S*)-phenylalanine was obtained in yield of 0.277 g (61%) with 99% *ee* as colorless crystals; $[\alpha]^{24}_D$ -35.8º (*c* 1.00, H_2O). ^1H NMR (D_2O, 400 MHz) δ: 7.2-7.4 (m, 5H), 4.1 (dd, 1H, *J* = 7.7, 5.4 Hz), 3.3 (dd, 1H, *J* = 14.6, 5.4 Hz), 3.1 (dd, 1H, *J* = 14.6, 7.8 Hz). MS m/z: calcd for $C_9H_{12}N_1O_2$ $[M+H]^+$ 166.0863; found, 166.0885.

Results and discussion

Co-expression of the mutant ACL racemase and D-amino acid amidase genes in* E. coli *cells.

E. coli transformant co-expressing mutant ACL racemase and DaaA was constructed (Table 3-1) and used as biocatalyst for DKR of α-amino acid amide to (*R*)-α-amino acid. Both enzymes were efficiently expressed such that enzyme activity of mutant ACL racemase and DaaA from this recombinant *E. coli* were 0.210 and 25.9 U/ml culture, in the optimum cultivation condition (37ºC for 24 h without IPTG), respectively. The amount of both enzymes in recombinant *E. coli* were estimated to be 5% of total soluble protein, respectively, however, these enzyme activities in 1 ml culture were not balanced because the specific activity of DaaA (approximately 1,100

Umg^{-1}) was about 100 times higher than the mutant ACL racemase (approximately 10 Umg^{-1}).

Table 3-1. Plasmids used and constructed in this study.

Plasmids	Relevant characteristics	References
pET15ACL	pET15b derivative containing ACL racemase gene from *Achromobacter obae*	77
pACLmut	pUC18 derivative containing mutant ACL racemase gene with two amino acid substitutions	Not published
pDaa-BFB40	pUC19 containing BFB40 gene. BFB40 is thermostable mutant D-amino acid amidase from *O. anthropi* SV3	18
pBD3	pUC19 derivative containing L-amino acid amidase gene from *B. diminuta*	15
pDBFB40	pSTV29 derivative containing BFB40 gene	In this work
pLaaABd	pSTV29 derivative containing L-amino acid amidase gene from *B. diminuta*	In this work

Cell-free extract of *E. coli* co-expressing the mutant ACL racemase and DaaA catalyzed the (*S*)-phenylalaninamide HCl (100 mM) as a substrate with higher yield and ee% compared with *E. coli* co-expressing natural ACL racemase and DaaA (Figure 3-1). The latter one caused only slight increase of (*R*)-phenylalanine and achieved accumulation of only 7.3 mM for 24 h, while natural L-amino acid amidase from *E. coli* had great influence on an unfavorable production of 13.0 mM (*S*)-phenylalanine. *E. coli* co-expressing mutant ACL racemase and DaaA quickly converted (*S*)-phenylalaninamide (100 mM) into (*R*)-phenylalanine (99.4 mM) with 99% ee in the cell-free extract when incubated for 6 h and naturally expressed L-amino acid amidase from *E. coli* hardly affected the enantiomeric purity.

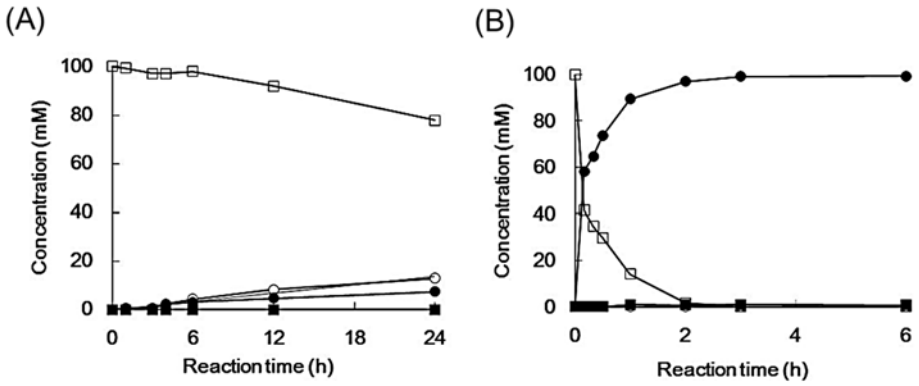


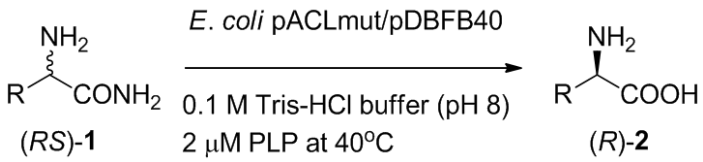
Figure 3-1. Time course for the production of (*R*)-phenylalanine from (*S*)-phenylalaninamide using *E. coli* pACL60/pDBFB40 (A) or *E. coli* pACLmut/pDBFB40 (B).

Symbol: (*R*)-Phenylalanine (●), (*S*)-phenylalanine (○), (*R*)-phenylalaninamide (■), (*S*)-phenylalaninamide (□)

Enzymatic conversion of several (*R*)-amino acids from the corresponding (RS)-amino acid amides.

The yields and enantiomeric excesses were measured for (*R*)-amino acids from several substrates (Table 3-2). (*RS*)-**1e**, (*RS*)-**1f**, (*RS*)-**1g**, (*RS*)-**1h**, and (*RS*)-**1i** were efficiently converted to the corresponding (*R*)-α-amino acids. (*RS*)-**1l** (50 mM) was quickly converted to (*R*)-**2l** (47.9 mM) in 4 h at 40°C but (*RS*)-**1l** (100 mM) inhibited the activity of *E. coli* co-expressing mutant ACL racemase and DaaA so that (*R*)-**2l** (87.8 mM) was produced after 12 h and with an optical purity of 77% ee. (*R*)-**2j** and (*R*)-**2k** were hardly produced with high yield and optical purity by DKR because the mutant ACL racemase showed difficulty in racemizing these halogen-containing substrates. To inhibit natural L-amidase from *E. coli*, cell-free extract was treated with 50°C for 10 min and used for the DKR of (*RS*)-**1c**, (*RS*)-**1j**, and (*RS*)-**1k.** The yield and optical purity of (*R*)-**2j** were dramatically increased up to 99% and 95% ee, whereas the optical purity of (*R*)-**2c** and (*R*)-**2k** were rather decreased. On the other hand, the

Table 3-2. Conversion of (*RS*)-α-amino acid amides to (*R*)-α-amino acids with *E. coli* pACLmut/pDBFB40.



Substrate R	Concentration [mM]	Reaction time [h]	Yield[a] [%]	E. e.[b] [% *ee*]
C_6H_5 (**1a**)	20	24	>99	88
4-F-C_6H_4 (**1b**)	20	24	>99	83
4-Cl-C_6H_4 (**1c**)	20	24	>99	16
4-CH_3-C_6H_4 (**1d**)	20	36	>99	-60
$C_6H_5CH_2$ (**1e**)	100	6	>99	99
4-OH-$C_6H_4CH_2$ (**1f**)	100	6	>99	>99
2-F-$C_6H_4CH_2$ (**1g**)	100	8	>99	93
3-F-$C_6H_4CH_2$ (**1h**)	100	8	>99	95
4-F-$C_6H_4CH_2$ (**1i**)	100	8	>99	97
4-Cl-$C_6H_4CH_2$ (**1j**)	40	24	>99	73
4-Br-$C_6H_4CH_2$ (**1k**)	30	24	>99	37
$C_6H_5CH_2CH_2$(**1l**)	100	12	>99	77

The reaction (1 ml) was carried out at 40°C by using cell-free extract (5 ml culture cell) of *E. coli* pACLmut/pDBFB40 containing 100 mM Tris-HCl buffer, pH 8.0, 2 μM PLP, and substrate. Reactions were not optimized except in (*RS*)-**1e**. [a],[b] The yielded and enantiomeric excess were determined by chiral HPLC.

substrate specificity of D-amino acid amidase purified with a His-tag column from *E. coli* was examined. Reaction mixture contained 100 mM Tris-HCl buffer, pH 8.0, 40 mM racemic substrate, and an appropriate amount of the purified enzyme. The specific activity towards (*RS*)-**1b**, (*RS*)-**1c**, (*RS*)-**1g**, (*RS*)-**1h**, (*RS*)-**1i**, (*RS*)-**1j**, and (*RS*)-**1k** were 2.30, 0.47, 4.58, 6.26, 3.69, 21.1, and 24.0% of the activity towards phenylalaninamide (1520 Umg^{-1}), respectively. While an analog with electron-donating substituent such as (*RS*)-**1d** was not a substrate for the recombinant enzyme and it was accepted by the natural amidase from *E. coli* with a higher formation of (*S*)-**2d** than (*R*)-**2d**. DaaA activity was not only influenced by the size of the side chain of the substrate, but also by the position of substituent of phenyl ring, while the strict *R*-stereoselectivity was

always maintained by these substitutions.

Enzymatic synthesis of (R)-2e, (R)-2f and (R)-2g using* E. coli *co-expressing mutant ACL racemase and DaaA (pACLmut/pDBFB40).

(*R*)-**2e,** (*R*)-**2f** and (*R*)-**2g** were synthesized by *E. coli* transformant co-expressing mutant ACL racemase and DaaA in the optimum condition (100 mM Tris-HCl buffer, pH 8.0, 40°C). (*RS*)-**1e** was added to the reaction mixture intermittently with portions of 0.2 g for four times, because mutant ACL racemase was indicated to be subject of substrate inhibition at more than 100 mM substrate concentration. Finally, the concentration of (*R*)-**2e** reached 395 mM in the reaction mixture with an optical purity of 99% ee (Figure 3-2 (A)).

(*R*)-**2f** and (*R*)-**2i** showed very low solubility in the reaction mixture at neutral pH, so that the product was quickly precipitated and efficiently shifting the reaction equilibrium toward the product formation. After 24 h, 356 mM of (*R*)-**2f** with 96% optical purity was produced. As a result, (*R*)-**2f** (isolation yield 73.9% and 99.9% ee) was synthesized from 0.4 g of (*RS*)-**1f** (369 mM) (Figure 3-2 (B)). Moreover, non-natural α-amino acid (*R*)-**2i**, is a raw material for peptidomimetic research [78], can also

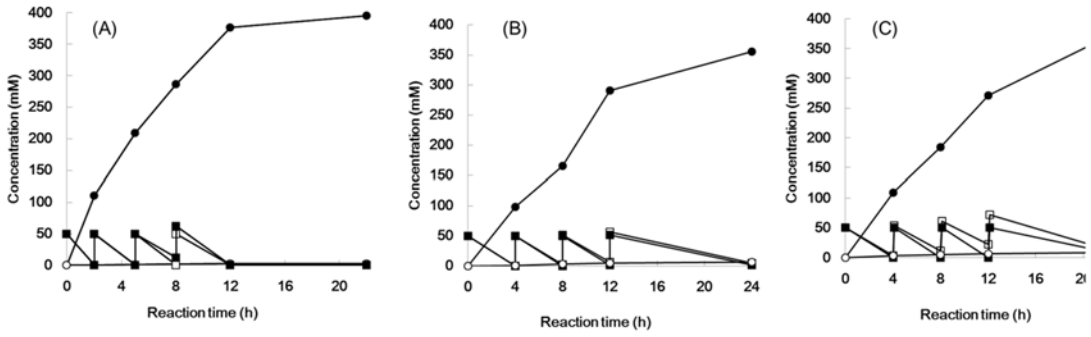


Figure 3-2. Enzymatic synthesis of (*R*)-**2e**, (*R*)-**2f** and (*R*)-**2g** using *E. coli* co-expressing mutant ACL racemase and DaaA. Symbol: (*R*)-acid (●), (*S*)-acid (○), (*R*)-amide (■), (*S*)-amide (□)

be synthesized from 0.44 g (*RS*)-**1i** (400 mM) in similar procedure (isolation yield 73% and >99% ee) (Figure 3-2 (C)).

Enzymatic synthesis of (S)-2e using* E. coli *transformant co-expressing mutant ACL racemase and recombinant L-amino acid amidase.

In this study, the author synthesized high concentration of (*S*)-**2e** from (*RS*)-**1e** by DKR using recombinant *E. coli* co-expressing mutant ACL racemase and recombinant L-amino acid amidase in optimum conditions (100 mM KPB, pH 7.0, 40ºC). *E. coli* JM109 harboring pACLmut and pLaaABd (Table 3-3) was cultivated in LB medium supplemented with ampicillin (80 μg/ml), and chloramphenicol (30 μg/ml) at 37ºC for 24 h under optimum conditions. The expression of mutant ACL racemase and L-amino acid amidase by *E. coli* pACLmut/pLaaABd (0.95 Umg^{-1} for mutant ACL racemase activity and 8.41 Umg^{-1} for $LaaA_{Bd}$ activity) were at the same level of the homogeneous expression of ACL racemase in *E. coli* pACLmut (1.0 Umg^{-1}), but lower than the $LaaA_{Bd}$ in *E. coli* pBD3 (25.9 Umg^{-1}) [15], respectively. Optically pure (*RS*)-**2e** with 61% isolation yield was synthesized in 24 h from (*RS*)-**1e** (400 mM) using cell-free extract of 25 ml culture (Figure 3-3). $LaaA_{Bd}$ acted on broad range of (*S*)-α-amino acid amides as substrates [15], preferentially on aromatic substrates. On the other hand, the enzyme showed a little amount of (*R*)-phenylalaninamide hydrolyzing activity forming (*R*)-phenylalanine. However, (*R*)-phenylalanine was synthesized only at 10.5 mM and easily removed by recrystallization. This result indicated that *E. coli* pACLmut/pLaaABd efficiently produced optically pure (*S*)-**2e** from (*RS*)-**1e**. The substrate specificity of the biocatalyst, *E. coli* pACLmut/pLaaABd, is summarized under Table 3-2. (*RS*)-**1f**-**1i** was rapidly and efficiently transformed into (*S*)-**2f**-**2i** with an optical purity of 90-98% ee

and 99% yield. Non-natural amino acid known as (*S*)-**2l** is an important chiral intermediate to synthesize a variety of pharmaceuticals including angiotensin converting enzyme inhibitors, β-lactam antibiotics, acetylcholine esterase inhibitors and neutral endopeptidase inhibitors. (*S*)-**2l** was also continuously precipitated in the reaction mixture and efficiently synthesized with >99% yield and 98% ee in 12 h.

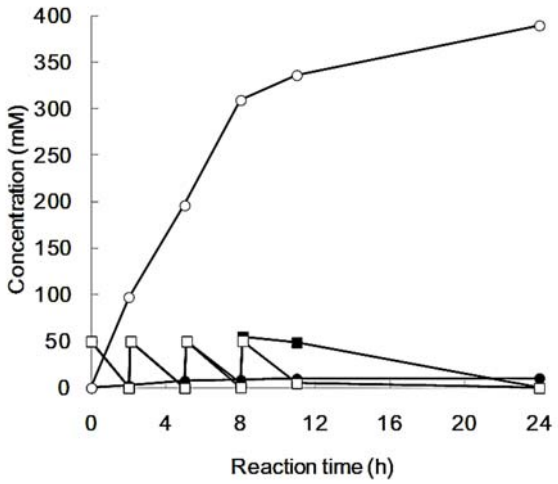
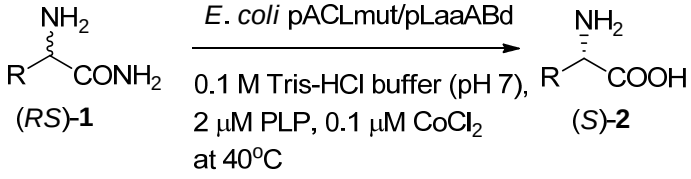


Figure 3-3. Time course for the enzymatic synthesis of (*S*)-phenylalanine using *E. coli* pACLmut/pLaaABd.

Symbol: (*R*)-acid (●), (*S*)-acid (○), (*R*)-amide (■), (*S*)-amide (□)

Table 3-3. Conversion of (*RS*)-α-amino acid amides to (*S*)-α-amino acids with *E. coli* pACLmut/pLaaABd.



Substrate R	Concentration [mM]	Reaction time [h]	Yield[a] [%]	E. e.[b] [% *ee*]
C_6H_5 (**1a**)	40	24	>99	>99
4-F-C_6H_4 (**1b**)	40	24	>99	95
4-Cl-C_6H_4 (**1c**)	20	24	>99	87
4-CH_3-C_6H_4 (**1d**)	20	36	85	96
$C_6H_5CH_2$ (**1e**)	100	4	>99	92
4-OH-$C_6H_4CH_2$ (**1f**)	100	6	>99	95
2-F-$C_6H_4CH_2$ (**1g**)	100	8	>99	97
3-F-$C_6H_4CH_2$ (**1h**)	100	8	>99	85 (91)
4-F-$C_6H_4CH_2$ (**1i**)	100	8	>99	98
4-Br-$C_6H_4CH_2$ (**1j**)	50	36	>99	84 (88)
4-Br-$C_6H_4CH_2$ (**1k**)	40	36	>99	50 (61)
$C_6H_5CH_2CH_2$(**1l**)	100	12	>99	98

The reaction (1 ml) was carried out at 40°C by using cell-free extract (5 ml culture cell) of *E. coli* pACLmut/pLaaABd (1.5 U of mutant ACL racemase and 9.2 U of $LaaA_{Bd}$) containing 100 mM KPB, pH 7.0, 2 μM PLP, 0.1 mM $CoCl_2$, and substrate. Reactions were not optimized except in (*RS*)-**1e**. Komeda et al. reported that $LaaA_{Bd}$ was very stable up to 60°C for 20 min. Therefore, cell-free extract of *E. coli* pACLmut/pLaaABd was treated with 60°C for 10 min and the treated enzyme was used for DKR of (*RS*)-**1h**, **1k** and **1l**. These optical purities were showed in parenthesis.

[a], [b] The yielded and enantiomeric excess were determined by chiral HPLC.

*Synthesis of (*R*)-2e from (*RS*)-phenylalaninonitrile using nitrile hydratase and* E. coli *pACLmut/pDBFB40.*

In the previous chapter, the author have successfully synthesized aliphatic (*R*)- and (*S*)-α-amino acids such as α-aminobutyric acid from (*RS*)-α-aminobutyronitrile by DKR using purified non-stereoselective nitrile hydratase (NHase), (*R*)- or (*S*)-

stereoselective amino acid amidase and ACL racemase. [59] When (*RS*)-phenylalaninonitrile (10 mM) was used as aromatic substrate, (*S*)-phenylalanine was produced in 12 h (yield 99%, 97% ee), although it was difficult to produce (*R*)-phenylalanine. In this study, sequential conversion of (*RS*)-phenylalaninonitrile to (*R*)-phenylalanine was performed. Since NHase and ACL racemase were inhibited by the α-aminonitrile, the substrate needed to be quickly and completely converted to the α-amino acid amide by the NHase. Finally, the author successfully carried out fed-batch reaction. In the initial 5 min, (*RS*)-phenylalaninonitrile (50 μmol) was quickly converted to racemic phenylalaninamide, and then (*R*)-phenylalaninamide was simultaneously hydrolyzed to (*R*)-phenylalanine by DaaA. After 30 min, further 50 μmol of the substrate was added to the reaction mixture and it was quickly converted to (*R*)-phenylalaninamide and ultimately to (*R*)-phenylalanine. Remaining (*S*)-phenylalaninamide was racemized by the mutant ACL racemase, and after 4 h, (*RS*)-2-phenylalaninonitrile (100 mM) was completely converted to (*R*)-phenylalanine with 95% yield and 90% ee (Figure 3-4). This one pot synthesis also represents a new synthetic approach to phenylalanine derivatives, which serve as interesting intermediate compounds for the synthesis of chiral compounds.

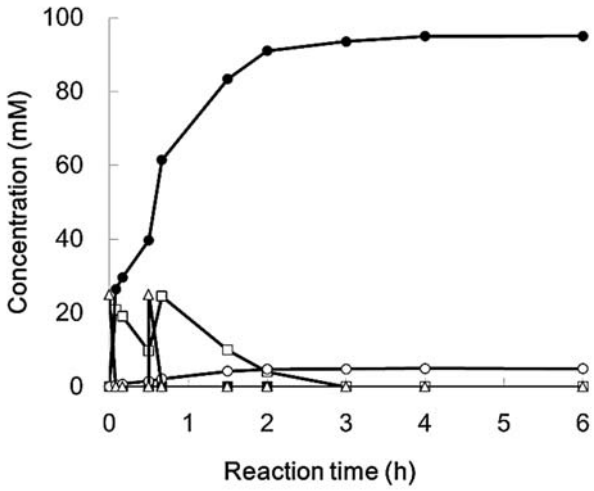


Figure 3-4. Time course for the enzymatic synthesis of (*R*)-phenylalanine from (*RS*)-phenylalaninonitrile by DKR.

Symbol: (*R*)-nitrile (▲), (*S*)-nitrile (Δ), (*R*)-acid (●), (*S*)-acid (○), (*R*)-amide (■), (*S*)-amide (□)

Chapter IV

Tailoring D-amino acid oxidase from the pig kidney to *R*-stereoselective amine oxidase and its use in deracemization of α-methylbenzylamine

The deracemization of racemic amines to form chiral amines using *S*-stereoselective amine oxidases (AOx) has recently been attracting attention. However, *R*-stereoselective AOx suitable for deracemization have not yet been identified. The purpose of the present study was evolving an R-stereoselective AOx from porcine kidney D-amino acid oxidase (pkDAO) and subsequently use it in the deracemization of racemic amine.

Engineered pkDAO with markedly changed substrate specificity toward (*R*)-amines was obtained by directed evolution. The mutant enzyme exhibited a high preference toward the substrate α-methylbenzylamine (MBA), and was used to synthesize (*S*)-amine through deracemization.

The findings of the present study indicate that further investigations on the structure-activity relationship of AOx are warranted and also provide a new tool for biotransformation in organic synthesis.

Experimental section

Enzyme assay.

Oxidase activity was routinely assayed at 30ºC by measuring the formation of a quinone pigment following absorbance at 505 nm with an absorption spectrometer. The reaction mixture contained 100 mM K_2HPO_4/KH_2PO_4 buffer (KPB, pH 8.0), 1.5 mM 4-aminoantipyrine, 2 mM phenol, 2 unit horseradish peroxidase, 10 mM substrate, and enzyme.

Analytical methods.

The chiralities of 1, 2, 3, and 4 were analyzed by HPLC (Shimadzu, Kyoto, Japan) using a Crownpak CR(+) column (Daicel Co., Japan) with 60 mM $HClO_4$/5% MeOH solution. UV detection was performed at 210 nm.

***Constraction of pDAO and expression of pkDAO gene in* E. coli.**

Assemble PCR.

The pkDAO gene is composed of 1050 nucleotides. Thirty-three oligonucleotides were designed on the basis of the nucleotide sequence of the pkDAO gene.

These oligonucleotides (Table 4-1) were combined and assembled by PCR. The reaction mixture (50 μl)for PCR contained KOD-plus- buffer, 0.2 mM dNTP, 1.5 mM $MgSO_4$, 0.02 pmol of each oligonucleotide and 1 U KOD -plus- polymerase. The PCR program consisted of 35 cycles at 94ºC for 30 s, 50ºC for 30 s, and 72ºC for 90 s. Approximately 1000 bp of the PCR product were isolated from the agarose gel and the isolated product gene was amplified in a 2^{nd} PCR by using the oligonucleotides DAO1 and DAO33 as outside primers. The amplified PCR product was analyzed using the ABI

PRISM 310 Genetic analyzer. A homology search was performed with the sequence similarity searching program BLAST and the ClustalW method was used to align the sequence. The genetyx software system (Software Development Co., Tokyo, Japan) was used for computer analysis of the nucleotide sequences and deduced amino acid sequences.

Construction of the expression vector pDAO.

The gene for the pkDAO was amplified by PCR with a primer set 5'-TTTGAATTCTAAggaggaCTAGCTCATGCGTGTGGTGGTGATT-3' and 5'-AATAAGCTTTCAGAGGTGGGATGGTGGCAT-3' (restriction site, Shine-Dalgarno sequence, and initiation codon are shown in underline, lowercase and bold letters, respectively) using the synthesis of the pkDAO gene as a template. The amplified fragment was cloned between the *Bam*HI and *Hind*III sites of pUC18 to produce pDAO.

The author constructed pDAO expression plasmids and transformed *E. coli* JM109 to express active pkDAO in *E. coli* JM109. Recombinant *E. coli* was grown in LB medium containing ampicillin (80 μg/ml) and 1 mM IPTG at 37°C for 24 h.

Table 4-1. Oligonucleotides synthesized based on the nucleotide sequence of pkDAO gene.

Nucleotide sequence	
atgcgtgtggtggtgattggagcaggcgtcattgggctgtccactgccc	DAAO1
gcgctaccactctgtcctgcagcccctcgatgtgaaggtctacgcagac	DAAO2
tcaccaccactgatgtagctgctggcctctggcagccctacacctctga	DAAO3
caggaggcgaactggaaccaacagaccttcaactatctcctgagtcaca	DAAO4
tgctgcaaacatgggtctgaccccagtctcaggctacaacctcttccgt	DAAO5
acccttactggaaagacatggtcctgggattccgaaagctgactcccag	DAAO6
tttcctgattatagatatggctggttcaacacatccctgattctggagg	DAAO7
acagtggctgacagaaaggttaactgagaggggagtgaaattcttcctg	DAAO8
cttttgaggaggtggcaagaggtggcgctgatgtgattatcaactgcac	DAAO9
ggggtgctgcaaccggatcccctgctgcagccaggccgggggcagatca	DAAO10
cccttggctgaagaacttcattatcacccatgacctagagagaggcatc	DAAO11
acatcattccagggctgcaggcagtgacacttggaggcaccttccaggt	DAAO12
gagataaataatatccaggaccacaacaccatctgggaaggctgctgca	DAAO13
actgaaggatgcaaaaattgttggtgaatatactggcttccggccagta	DAAO14
ggctagaaagagaacagcttcgctttggatcttcaaacacagaggtcat	DAAO15
catggaggctatgggctcaccatccactggggctgtgccctagaggtgg	DAAO16
gaaagtcctggaagaaaggaatttgctcacaatgccaccatcccacctctga	DAAO17
aggacagagtggtagcgctcgtggatgcagagggcagtggacagcccaa	DAAO18
ctacatcagtggtggtgaacggggtgaagcggtctgcgtagaccttcac	DAAO19
gttccagttcgcctcctgtgggttgctgggctcagaggtgtagggctgc	DAAO20
agacccatgtttgcagcattgggagaaccgatgtgactcaggagatagt	DAAO21
tgtctttccagtaagggtccggaacagcttcacggaagaggttgtagcc	DAAO22
atatctataatcaggaaacatgtccagttctctgggagtcagctttcgg	DAAO23
ctttctgtcagccactgtaggtacttccttccctccagaatcagggatg	DAAO24
ttgccacctcctcaaaagattccaccttcctcaggaagaatttcactcc	DAAO25
atccggttgcagcaccccagcccacaccccagtgcagttgataatcaca	DAAO26
aagttcttcagccaaggggcatccaccttaatgatctgcccccggcctg	DAAO27
gcagccctggaatgatgtatggagagttgtagatgcctctctctaggtc	DAAO28
ctggatattatttatctcattccagttccccacctggaaggtgcctcca	DAAO29
atttttgcatccttcagtgtgggctccagtctgcagcagccttcccaga	DAAO30
gctgttctctttctagccgaacctgggggcgtactggccggaagccagt	DAAO31
gagcccatagcctccatggccatagttgtggatgacctctgtgtttgaa	DAAO32
ctttcttccaggactttcccaaagagcttggccacctctagggcacagc	DAAO33

Site-directed mutagenesis of pkDAO.

Site-directed mutagenesis was carried out with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Plasmid pDAO containing the gene coding for the pkDAO was extracted from *E. coli* JM109/pDAO and used as a template in saturation mutagenesis. The reaction mixture consisted of 5 μl 10 x reaction buffer, 1 μl of dNTP mix, 41 μl of double-distilled water, 1 μl of 2.5 U/μl PfuTurbo DNA polymerase, 1 μl (125 ng/μl) of sense and antisense primers (Table 4-2), and 1 μl of 50 ng/μl plasmid pDAO as template DNA. Sixteen cycles were preformed, with each consisting of a denaturing step at 95°C for 30 s (first cycle at 95°C for 30 s), annealing step at 55°C for 1 min, and elongation step at 68°C for 4 min. The product was treated with 10 U of *Dpn*I at 37°C for 3 h, and then used to transform *E. coli* JM109.

Table 4-2. PCR primer set for mutation study.

Nucleotide sequence	
agaggcatctacaactctccannnatcattccagggctgc	Y228XF
tgaatatactggcttcnnnccagtacgccccca	R283XF
gcagccctggaatgatnnntggagagttgtagatgcctct	Y228XR
tgggggcgtactggnnngaagccagtatattca	R283XR

Purification of recombinant pkDAO from* E. coli *JM109 pDAOmut.

The mutant pkDAO (Y228L, R283G) was purified 18-fold with a yield of 46.9% from the cell-free extract by solid ammonium sulfate (20% to 35% saturation), and DEAE-Toyopearl 650M and Butyl-Toyopearl 650M column chromatographies (Table 4-4). The final preparation gave a single band on SDS-PAGE.

The purification of the wild-type pkDAO was performed in the same manner and the specific activity of final preparation toward (*R*)-phenylalanine (10 mM) was calculated

as 4.9 U/mg.

Stereoinversion reaction of (R)-3a.

The stereoinversion reaction was performed in 100 mM KPB (pH 8.0), 20 mM chemical reductant, 1 mM (*R*)-**3a**, and enzyme.

Preparative scale synthesis of (S)-3a by deracemization.

The identity of (*S*)-**3a** formed by deracemization using the purified pkDAO mutant was confirmed by its isolation. The reaction mixture (400 mL) contained 40 mmol KPB buffer (pH 8.0), 40 mmol $NaBH_4$, 2.0 mmol (0.24 g) (*RS*)-**3a**, and 300 U purified enzyme. The mixture was then incubated at 30°C for 3 h. The reaction mixture was adjusted to pH 12 with 6 N NaOH and the product was extracted 4 times with dichloromethane. The organic phase was evaporated in vacuo and the remaining product was subsequently purified by silica column chromatography (AcOEt: MeOH = 1:1). The optical purity of the isolated (*S*)-**3a** was more than 99% ee with an isolation yield of 64.5% (0.156 g, 1.29 mmol); optical rotations were measured on a ATAGO AP-300 automatic polarimeter (Atago Co., Tokyo, Japan). $[\alpha]^{26}_D$ -29.4° (c 1.02, MeOH) (ref. 79, $[\alpha]^{20}_D$ -25.7° (c 0.98, MeOH), ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.29 (d, 3H, *J* = 6.6 Hz), 1.61 (s, 2H), 4.01 (q, 1H, *J* = 6.6 Hz), 7.11-7.23 (m, 5H). MS (microTOF) m/z: calcd for $C_8H_{12}N_1$ $[M+H]^+$ 122.0964; found, 122.0992.

Crystallization of the D-amino acid oxidase mutant.

The pkDAO mutant sample was concentrated to more than 50 mg/mL, and 1.0 M ammonium sulfate solution was added into the sample. The sample was incubated for 1

hour at 277K, and the appearance of precipitation was discarded by centrifugation. The lysate was used for crystallization. A crystallization trial was performed at 293K using the sitting vapor diffusion method. Drops were prepared by mixing 2.0 μL of the protein solution with 1.0 μL of the reservoir solution. Crystallization condition screening was performed using a commercially available screening kit, and block like crystals were obtained using a reservoir solution consisting of 30% polyethylene glycol (PEG) 4000, 0.1 M Tris-HCl pH 8.5, and 0.2 M lithium sulfate.

X-ray data collection.

pkDAO mutant crystals were soaked in the reservoir solution, which contained 20% PEG400 and 10mM (*R*)-methylbenzylamine, for 30 min prior to the collection of X-ray diffraction data. The soaked crystal was mounted and flash-cooled under a liquid nitrogen stream (100K). Diffraction data was collected at BL17A (KEK). The indexing and integration of diffraction data were performed by HKL2000[80], and scaling was performed by Scalepack.[80] The initial phase was determined by Molrep[81] in the CCP4 program suit[82] using the crystal structure of pkDAO from the porcine liver (PDB ID: 1VE9) as template. Model building and structure refinement were performed by Coot[83] and Refmac[84], respectively. All structural figures were prepared by PyMol[85].

Results and Discussion

The occurrence of AOx with *R*-stereoselectivity has not been reported previously, whereas *S*-stereoselective flavin-dependent AOx belonging to the AOx family proteins, including L-amino acid oxidase (LAO), polyamine oxidase, and spermine oxidase, has. The author targeted the DAO family of proteins, especially pkDAO as a starting enzyme for directed evolution, because the author observed from their primary structures that the DAO family of proteins, including DAO, glycine oxidase, and sarcosine oxidase may have diverged from a common ancestral protein; therefore, the potential protein structure of *R*-stereoselective AOx should resemble that of DAO. Therefore, the author speculated that it may be possible to tailor make AOx from the typical DAO group of enzymes such as pkDAO.

pkDAO was identified as the first mammalian flavoprotein catalyzing the oxidative deamination of α-amino acids with strict *R*-stereoselectivity to form the corresponding α-keto acids, ammonia, and hydrogen peroxide, while it does not act on simple amine compounds. A previous study revealed the structure of flavin-dependent pkDAO complexed with an inhibitor benzoate (PDB: 1VE9).[86] The overall structure of pkDAO was markedly different from that of the AOx family of proteins. On the other hand, the substrate-binding site of pkDAO and LAO were shown to be linked to each other in a “mirror image” relationship, with their catalytic mechanisms having many similarities.[58] The carboxylate group of benzoate interacts with the guanidine moiety of Arg283 and the hydroxyl oxygen of Tyr228 acts as the carboxylate binding site in cooperation with Arg283 in pkDAO. This arginine residue is conserved in several D- or L-amino acid oxidases from different sources. Therefore, the residues Tyr228 and Arg283 in the catalytic site were chosen as the targets of mutation to improve substrate

specificity. The pkDAO gene was synthesized by assembly PCR and expressed in *E. coli*. The single saturation mutagenesis of residues Tyr228 and Arg283 was performed and the resulting mutant libraries were screened by the oxidation of (*RS*)-α-MBA ((*RS*)-**3a**). Positive clones were determined by a colorimetric assay to measure amine oxidase activity. The positive mutant could not be obtained the positive mutant from the saturation mutagenesis library of Tyr228, while screening among saturation mutagenesis library of residue Arg283, twenty variants were obtained with oxidation activity toward (*RS*)-**3a**. The residue Arg283 was found to be altered to either Gly, Ala, or Cys among the positive mutant enzymes obtained. The mutants R283G, R283A, and R283C catalyzed the oxidation of the *R*-enantiomer of (*RS*)-**3a**. These mutants were used as parents for the second round of saturation mutagenesis of Tyr228 and screening. The resulting mutants such as Y228L/R283G, Y228L/R283A, and Y228L/R283C were obtained by screening for higher oxidative activity than that of the parents (Table 4-3).

Table 4-3. Comparison of the activities of the pkDAO variants.

	Relative activity (%)	
Variant pkDAO	(*R*)-Phe	(*R*)-MBA
Wild-type	100 (0.11 U/mg)	0
R283G	0	26
R283A	0	14
R283C	0	14
Y228L/ R283G	0	146
Y228L/ R283A	0	20
Y228L/ R283C	0	38
Y228L	29	0

The activity of (*R*)-Phenylalanine (Phe), corresponding to 0.11 Umg^{-1}, was taken as 100%.

The reaction mixture (total volume 1.0 mL) was composed of 100 mM KPB (pH 8.0), 10 mM substrate, and an appropriate amount of the cell-free extract.

Table 4-4. Summary of the purification of the mutant pkDAO (Y228L, R283G).

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	1800	2130	1.18	100
20-35% $AmSO_4$ fractionation	538	1450	2.68	68.1
DEAE-Toyopearl	127	1070	8.47	50.2
Butyl-Toyopearl	46.7	1000	21.5	46.9

10 mM (*R*)-α-Methylbenzylamine was used as a substrate.

These mutants also showed highly stereoselective oxidase activity toward *R*-enantiomer of (*RS*)-**3a** only. Finally, mutant pkDAO (Y228L/R283G) was selected for further investigation because it showed the highest activity toward (*RS*)-**3a**. The mutant pkDAO (Y228L/R283G) was purified (Table 4-4) and characterized from recombinant *E. coli* JM109. The specific activity of the purified enzyme was 21.5 Umg^{-1} using (*R*)-**3a** as a substrate and the enzyme showed strict *R*-stereoselective amine oxidase activity for (*RS*)-**3a**. This mutant lost its ability to catalyze the oxidation of (*R*)-amino acids such as phenylalanine, proline, methionine, and alanine. Its specific activity was four times higher than that of the purified wild-type pkDAO (4.9 U/mg, with (*R*)-phenylalanine as the substrate). The reaction profile of the kinetic resolution of 10 mM (*RS*)-**3a** to (*S*)-**3a** using the purified enzyme was shown in Figure 3A. The initial presence of (*R*)-**3a** in the reaction mixture was completely bolished within 2 h and the enantiomeric excess of the remaining (*S*)-**3a** reached 99%. This result indicated that the mutant enzyme is characterized as a novel stereoselective *R*-amine oxidase that could be applied to the production of chiral (*S*)-amine by kinetic resolution. Other mutants also showed complete *R*-stereoselective activity toward (*RS*)-**3a**. The mutant enzyme also exhibited

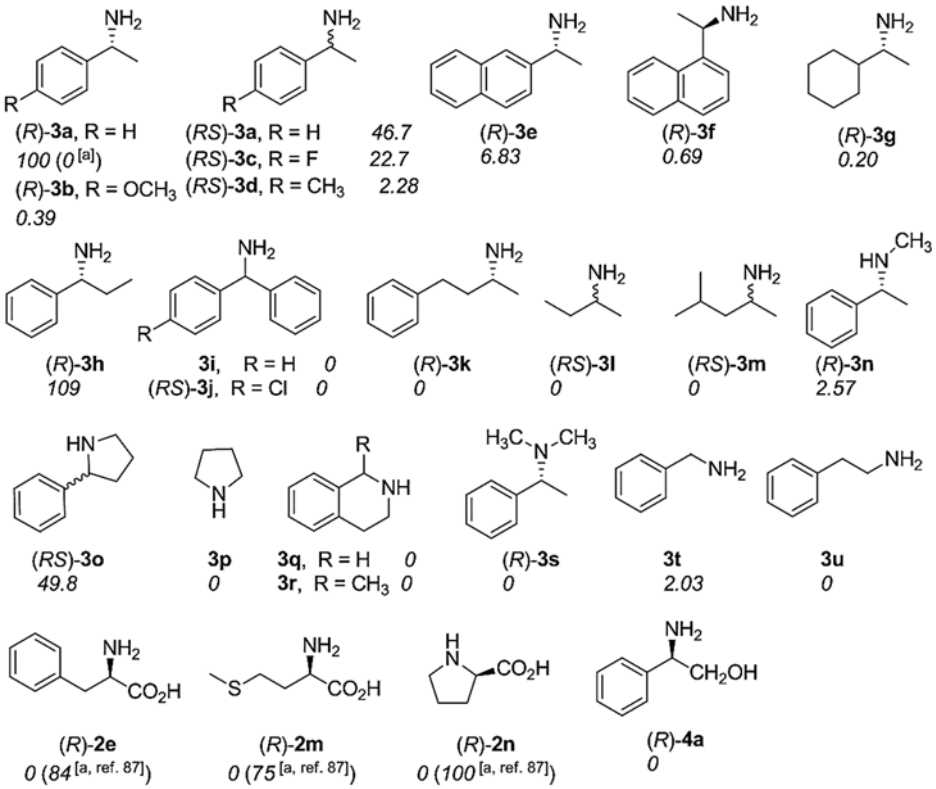


Figure 4-1. Substrate specificity of mutant pkDAO.

Enzyme activities were measured as described under "Experimental section". The activity for (*R*)-**3a** corresponding to 21.5 Umg^{-1} was taken as 100%.

The italic numbers below the structures indicate relative activity.

[a] Substrate specificity of wild-type pkDAO.

strong preference to MBA derivatives (especially, **3a**, **3h**, and **3o**), whereas the enzyme hardly oxidized chiral aliphatic primary amine (**3l** and **3m**), simple primary amines (**3t** and **3u**), or chiral secondary (*R*)-amines (**3n**) (Figure 4-1). However, (*S*)-amines (**3a-h, 3n**) were not the substrates. The stereoselectivity of the mutant pkDAO against (*RS*)-**3a**, (*RS*)-**3c**, and (*RS*)-**3d** was determined by chiral HPLC. Both the enantiomers of amino acids such as **2e**, **2m**, and **2n**, and glycine were not the substrates. A comparison of the mutant enzyme with the wild-type pkDAO showed that they had the same properties (optimum pH 9 and temp. 45°C), except for heat stability: the mutant enzyme was stable

at 55°C, while the wild-type enzyme was stable only up to 45°C [87] (Figure 4-2).

The wild-type pkDAO has been used in the deracemization of α-amino acid to form (*S*)-α-amino acid.[88-91] The Y228/R283G mutant enzyme was also capable of the stereoinversion of (*R*)-amine using a chemical reductant such as $NaBH_4$.

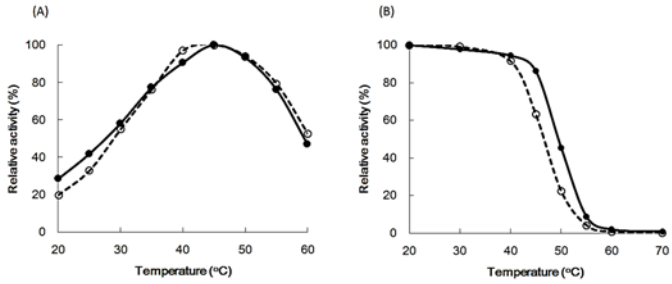


Figure 4-2. Optimum temperature (A) and heat stability (B) on the activity of wild and mutant pkDAO.

(A) Reactions were carried out at various temperature as described in Experimental section.

(B) Remaining activity of the enzymes were measured as described in Experimental section after incubation of enzymes at various temperature for 30 min.

The mutant enzyme lost its activity under the harsh deracemization reaction using $NaBH_4$ at high temperature. The activity of the enzyme was almost lost when it was incubated at 45°C for 30 min in the presence of 100 mM $NaBH_4$. Milder and more stable chemical reductants such as $NaCNBH_3$ and NH_3-BH_3 were previously shown to be suitable for use in deracemization reactions using AOx.[51-56, 90, 91] However, the conversion of 1 mM (*R*)-**3a** to (*S*)-**3a** was lower with $NaCNBH_3$ (yield: 8.5%) and NH_3-BH_3 (48%) than with $NaBH_4$ (96%). The use of $NaCNBH_3$ or NH_3-BH_3 led to the formation of unfavorable ketone or resulting alcohol by reduction of ketone as the main product. The preparative scale synthesis of (*S*)-**3a** from racemic **3a** by deracemization was performed at 30°C under the following optimum conditions, 100 mM KPB buffer (pH 8.0) containing 5 mM (*RS*)-**3a**, 100 mM $NaBH_4$, and 300 U purified enzyme. The typical time course of the deracemization reaction was shown in Figure 4-3B. Stereoinversion-induced conversion from (*R*)-**3a** to (*S*)-**3a** was quantitative and (*RS*)-**3a**

was completely converted to (*S*)-**3a** (99% ee) with no detectable by-product after a 3 h reaction. Approximately 35% of (*S*)-amine was lost in a purified process, and the isolation yield was 65%. To the best of knowledge, this is the first study to examine the synthesis of (*S*)-amine by the deracemization process using an (*R*)-stereoselective amine oxidase. This mutant enzyme is useful for producing the *S*-enantiomer of amine compounds by not only kinetic resolution, but also the deracemization process.

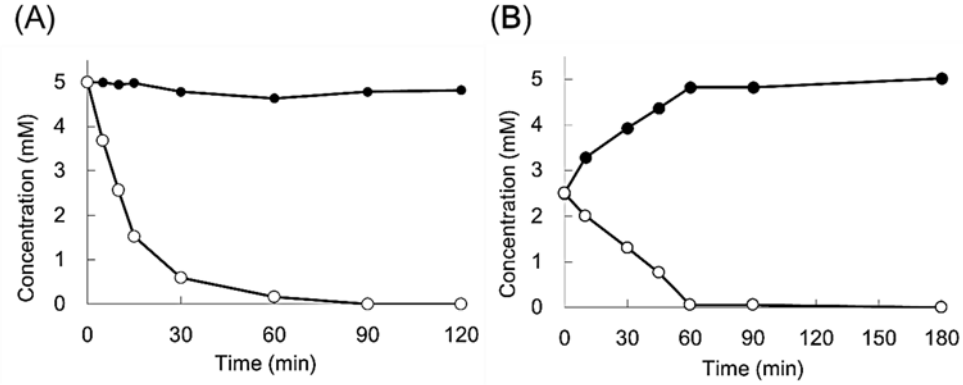


Figure 4-3. Time course of the kinetic resolution (A) and deracemization (B) of (*RS*)-**3a** using the mutant pkDAO (Y228L/R283G).

(A) The kinetic resolution of 10 mM (*RS*)-**3a** was carried out with 1.5 U mutant pkDAO in 100 mM KPB (pH 8.0) at 30°C. (B) The enzymatic conversion of (*RS*)-**3a** to (*S*)-**3a** by deracemization. The reaction mixture (total volume 1.0 ml) was composed of 100 mM KPB buffer (pH8.0), 5 mM (*RS*)-**3a**, 100 mM $NaBH_4$, and the mutant enzyme (0.5 U) at 30°C. The enzyme was added to initiate the reaction.

Symbols: (*S*)-**3a** (●) and (*R*)-**3a** (○).

A single amino acid change in the mutant (R283G) to a marked conversion in the substrate specificity of the enzyme from amino acid oxidase to amine oxidase. The author determined the crystal structure of pkDAO mutant (Y228L/R283G) (PDB: 3WGT). The crystal structure of the (*R*)-**3a** binding pkDAO mutant was determined at 1.88 Å (Table 4-5) and the active site structure was shown in Figure 4-3A. The electron

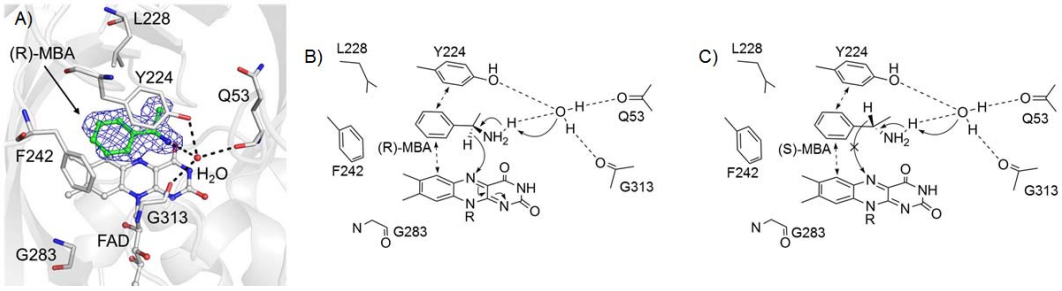


Figure 4-4. (A) The active site of the pkDAO mutant (Y228L/R283G) bound with (*R*)-MBA. The carbon atoms of (*R*)-**3a** ((*R*)-MBA in the figure) were colored in green. The carbon atoms of pkDAO and FAD were colored in gray. All hydrogen bonds were less than 3.4 Å. The $2F_o$-F_c difference Fourier maps (blue) were contoured at 0.8σ. (B) Proposed mechanism for the (*R*)-**3a** binding form for the pkDAO (Y228L/R283G) mutant. (C) Proposed mechanism for the (*S*)-**3a** binding form for the pkDAO (Y228L/R283G) mutant. The reaction mechanism for the mutant was depicted based on the already suggested reaction mechanism of the wild-type pkDAO.[92]

density of (*R*)-**3a** is confirmed on the *re*-side of FAD (Figure 4-4A). A hydrophobic cavity, which has enough space for the phenyl ring of (*R*)-**3a** to be inserted (Figure 4-5A) was created on the xylene ring of FAD in the Y228L/R283G mutant. In this location, the benzene ring of the substrate was sandwiched between the xylene ring of FAD and *p*-hydroxyl phenyl group of Tyr224 and formed π-π stacking interactions with each xylene ring and *p*-hydroxyl phenyl group of Tyr224 (Figure 4-5B). This is different from benzoate as an inhibitor binding form of the wild-type pkDAO in the crystal structure, the phenyl group of the benzoate is located on the uracil ring of FAD (Figure 4-5C). The marked change in the placement of the phenyl ring by the mutation (Figure 4-5A) gave *R*-selective amine oxygenase catalytic potency to the mutant by the following mechanism. Regarding (*R*)-**3a** binding, dehydrogenation could easily be performed because an α-hydrogen atom of the substrate was directed to the side of the N5 atom of FAD (Figure 4-4B). On the other hand, the α-hydrogen atom is directed to the side of Tyr224. The dehydrogenation could not be performed because the hydrogen atom is remote from the N5 atom of FAD (Figure 4-4C).

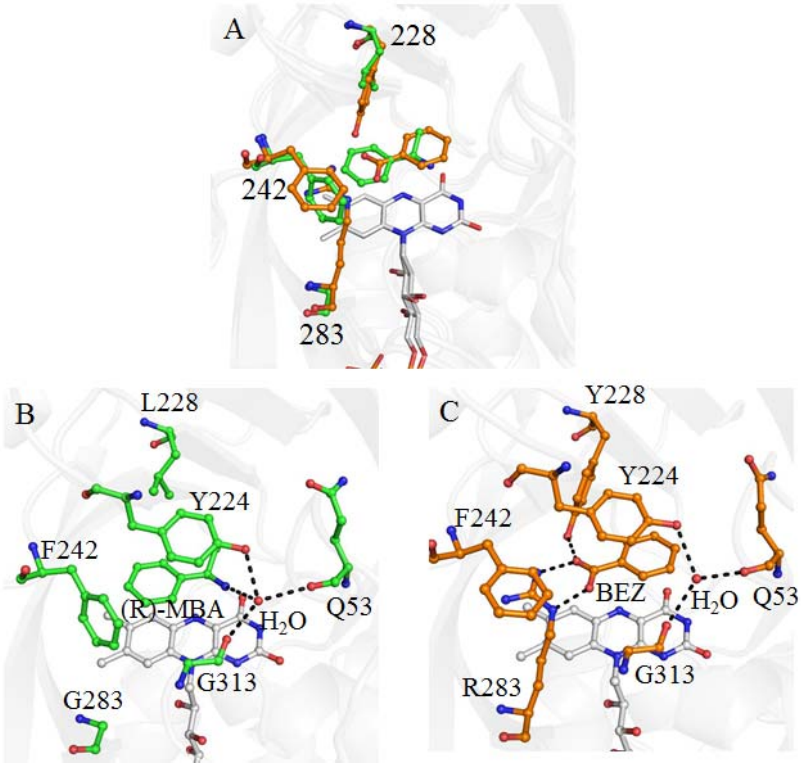


Figure 4-5. Active site structures of wild type (orange, PDB ID: 1VE9) and Y228L/R283G mutant (green) of the pkDAO.

A), superimposed structures of the wild type and the mutant. The mutation of Arg283 to Gly created a cavity on xylene ring of the FAD, and side chain of F242 rotated about 60 degree (x2 angle) to fill the cavity. B, C), substrate binding form of the mutant (B) and the wild type (C). All hydrogen bonds were less than 3.4 Å.

Table 4-5. Statistics of X-ray diffraction data collection for (*R*)-α-methylbenzylamine ((*R*)-MBA) binding pkDAO (Y228L/R283G) mutant.

	pkDAO (Y228L/R283G, (*R*)-MBA)
Space Group	$P2_12_12_1$
Unit cell parameters	
a (Å)	68.62
b (Å)	91.61
c (Å)	110.32
X-ray source	BL17A (KEK)
Wavelength (Å)	0.98
Resolution (Å)	34.1 – 1.87 (1.92 –1.87)
No. of reflection	286913
No. of unique reflection	56667
Completeness (%)	97.2
I/sig(I)	33.8 (6.5)
R_{merge}[a]	0.076 (0.387)
R[b]	0.191
R_{free}[c]	0.230
Geometry	
Bond length	0.011
Bond angle	1.672
RMSD of geometry	
Ramachandran outlier (%)	0.0
Ramachandran favored (%)	100
PDB code	3WGT

[a] $R_{merge} = \Sigma_h\Sigma_i|I_i(h)-\langle I(h)\rangle|/\Sigma_h I(h)$, where $I_i(h)$ is the ith measurement of reflection h, and $\langle I(h)\rangle$ is the mean value of the symmetry-related reflection intensities. Values in brackets are for the shell of the highest resolution.

[b] $R = \Sigma||F_o|-|F_c||/\Sigma|F_o|$, where F_o and F_c are the observed and calculated structure factors used in the refinement, respectively.

Conclusion

Chapter I

Five α-aminonitrile hydrolyzing microorganisms were isolated from soil samples. Strain 71D, a bacterium producing high NHase with low stereoselective toward α-aminobutyronitrile was selected and identified as *R. opacus*. NHase from *R. opacus* 71D was purified to homogeneity and its properties were characterized. This is the first characterization of the NHase acting on α-aminonitriles.

In this chapter, the author described the feasibility of achieving the sequential conversion of racemic α-aminonitriles to chiral amino acids in one pot enzymatic reaction by using purified NHase, ACL racemase and *R*- or *S*-stereoselective amidase. This is the first report of DKR of racemic α-aminonitriles to form chiral amino acids. This new method of DKR has a possibility to be developed to the large scale production of optically active α-amino acids.

Chapter II

The NHase gene from *R. opacus* 71D was cloned and expressed in *E. coli* JM109. The NHase activity in the cell of the *E. coli* harboring pNH2 was about 30 times higher than *R. opacus* 71D. In this study, non-natural (*R*)-α-aminobutyric acid was synthesized in a high concentration from racemic α-aminobutyronitrile using cell-free extracts of *E. coli* pNH2, *E. coli* pACL60 and *E .coli* pDAP1.

Chapter III

The mutant ACL racemase used in this chapter III showed a high catalytic efficiency toward phenylalaninamide compared with the natural one. In this study, the author has successfully synthesized natural and non-natural (*R*)-phenylalanine derivatives with commercial interests with excellent enantiomeric purities in high yield from the corresponding (*RS*)-α-amino acid amides by DKR using cell-free extract of *E. coli* co-expressing both of the D-amino acid amidase and mutant ACL racemase. (*S*)-Phenylalanine was also synthesized from (*RS*)-phenylalaninamide by cell-free extract of *E. coli* co-expressing two enzymes the L-amino acid amidase and mutant ACL racemase. Optically pure (*R*)-phenylalanine was obtained from (*RS*)-phenylalaninonitrile via (*RS*)-phenylalaninamide in one step by a combination of the cell-free extracts from recombinant *E. coli* encoding NHase as well as *E. coli* co-expressing D-amino acid amidase and mutant ACL racemase. This new DKR method could be very useful in the production of optically pure phenylalanine derivatives.

Chapter IV

The author demonstrated engineered pkDAO catalyzed oxidation activity toward several (*R*)-amines for the formation of their corresponding imines with high stereoselectivity, and this could not be performed by the wild-type pkDAO. Amino acid oxidases are not known to catalyze the oxidation of amines, not even L-amino acid oxidase, which belongs to the AOx protein family. On the other hand, the actions of AOx on amino acid have also not been examined yet. In spite of the different substrate specificities between amino acid oxidase and AOx, a single point mutation (R283G) in

pkDAO led to a marked change in the properties of D-amino acid oxidase into an amine oxidase and the mutant (Y228L/R283G) showed improved catalytic activity toward (*R*)-MBA. The crystal structure of the mutant enzyme revealed the recognition of (*R*)-MBA in the active site. The phenyl group of (*R*)-MBA may have been fit to a new hydrophobic cavity created by the mutation such that the α-hydrogen atom of (*R*)-MBA was directed to the side of the N5 atom of FAD. The author demonstrated that enantiopure (*S*)-MBA could be synthesized from racemic MBA by a deracemization process using an engineered enzyme with *R*-stereoselective amine oxidizing activity in the presence of a chemical reductant. This is the first study to identify a novel tailor-made flavin-containing *R*-stereoselective amine oxidase that is applicable to the production of chiral (*S*)-amine by deracemization.

Acknowledgements

The author wishes to express his sincere thanks to Professor Yasuhisa Asano, Toyama Prefectural University, for his continuous guidance and encouragement during the course of this work. He showed me different ways to approach a research problem and the need to be persistent to accomplish any goal. The author is also grateful to Associate professor Hidenobu Komeda and Assistant Professor Ken-ichi Fuhshuku, Toyama Prefectural University for their practical guidance and advice in carrying out this work.

It is a great pleasure to acknowledge the members of my thesis review committee: Professor Yasuo Kato and Professor Noriyuki Nakajima Toyama Prefectural University, and Professor Tadao Oikawa, Kansai University.

Thanks are due to Dr. Ryuji Hasemi, Mitsubishi Gass Chemical Co., Inc and Dr. Shogo Nakano, Toyama Prefectural University, JST, ERATO, Asano Active Enzyme Molecule Project for their much helpful collaborations.

The author is greatly appreciates kindness shown by the members of the laboratory for Enzyme Chemistry and engineering, Biotechnology Research Center, Toyama Prefectural University and JST, ERATO, Asano Active Enzyme Molecule Project.

During the course of this work, at Toyama Prefectural University (2005-2009), the author was supported by the Japan Student Services Organization (JSSO).

Last, but not least, the author thanks his family for their encouragements, supports, and tolerances.

References

[1] W. Leuchtenberger, K. Huthmacher, K. Drauz, *Appl*. *Microbiol*. *Biotechnol*. **2005**, *69*, 1-8.

[2] Y. Asano, A. Yamada, Y. Kato, K. Yamaguchi, Y. Hibino, K. Hirai, K. Kondo, *J*. *Org*. *Chem*. **1990**, *55*, 5567-5571.

[3] F. Paradisi, S. Collins, A. R. Maguire, P. C. Engel, *J*. *Biotechnol*. **2007**, *128*, 408-411.

[4] K. Vedha-Peters, M, Gunawardana, J. D. Rozzell, S. J. Novick, *J*. *Am*. *Chem*. *Soc*. **2006**, *128*, 10923-10929.

[5] N. J. Turner, *Curr*. *Opin*. *Chem*. *Biol*. **2011**, *15*, 234-240.

[6] Y. Asano, Y. Kato, *Biosci*. *Biotech*. *Biochem*. **1994**, *58*, 223-224.

[7] Y. Asano, K. Kishino, A. Yamada, S. Hanamoto, K. Kondo, *Recl*. *Trav*. *Chim*. *Pays-Bas* **1991**, *110*, 206-208.

[8] A. M. Macadam, C. J. Knowles, *Biotechnol*. *Lett*. **1985**, *7*, 865-870.

[9] T. C. Bhalla, A. Miura, A. Wakamoto, Y. Ohba, K. Furuhashi, *Appl*. *Microbiol*. *Biotechnol*. **1992**, *37*, 184-190.

[10] M. A. Wegman, U. Heinemann, F. Van Rantwijk, A. Stolz, R. A. Sheldon, *J*. *Mol*. *Catal*. *B: Enzymatic* **2001**, *11*, 249-253.

[11] M. A. Wegman, L. M. Van Langen, F. Van Rantwijk, R. A. Sheldon, *Biotechnol*. *Bioeng*. **2002**, *79*, 356-361.

[12] M. Hensel, S. Lutz-Wahl, L. Fischer, *Tetrahedron: Asymmetry* **2002**, *13*, 2629-2633.

[13] S. Servi, D. Tessaro, G. Pedrocchi-Fantoni, *Coord*. *Chem*. *Rev*. **2008**, *252*, 715-726.

[14] H. Komeda, H. Harada, S. Washika, T. Sakamoto, M. Ueda, Y. Asano, *Eur*. *J*. *Biochem*. **2004**, *271*, 1465-1475.

[15] H. Komeda, H. Hariyama, Y. Asano, *Appl*. *Microbiol*. *Biotechnol*. **2006**, *70*, 412-421.

[16] Y. Asano, A. Nakazawa, Y. Kato, K. Kondo, *J. Biol. Chem.* **1989**, *25*, 14233-14239.

[17] H. Komeda, Y. Asano, *Eur. J. Biochem.* **2000**, *26*, **7**2028-2035.

[18] H. Komeda, N. Ishikawa, Y. Asano, *J. Mol. Catal. B: Enzymatic* **2003**, *21*, 283-290.

[19] Y. Asano, S. Yamaguchi, *J. Am. Chem. Soc.* **2005**, *12*, **7**7696-7697.

[20] Y. Asano, S. Yamaguchi, *J. Mol. Catal. B: Enzymatic.* **2005**, *36*, 22-29.

[21] S. Yamaguchi, H. Komeda, Y. Asano, *Appl. Environ. Microbiol.* **2007**, *73*, 5370-5373.

[22] H. Yamada, Y. Asano, T. Hino, Y. Tani, *J. Ferment. Technol.* **1979**, *57*, 8-14.

[23] Y. Asano, T. Yasuda, Y. Tani, H. Yamada, *Agric. Biol. Chem.* **1982**, *46*, 1183-1189.

[24] Y. Asano, Y. Tani, H. Yamada, *Agric. Biol. Chem.* **1980**, *44*, 2251-2252.

[25] Y. Asano, K. Fujishiro, Y. Tani, H. Yamada, *Agric. Biol. Chem.* **1982**, *46*, 1165-1174.

[26] Y. Asano, *Nippon Nogeikagaku kaishi* **1991**, *65*, 1617-1626.

[27] H. Yamada, M. Kobayashi, *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1391-1400.

[28] Y. Fukuda, M. Fukui, T. Harada, Y. Izumi, *J. Ferment. Technol.* **1971**, *49*, 1011-1016.

[29] Y. Asano, Y. Kato, *FEMS Microbiol. Lett.* **1998**, *158*, 185-190.

[30] Y. Kato, Y. Asano, *J. Mol. Catal. B: Enzymatic* **1999**, *6*, 249-256.

[31] Y. Kato, K. Nakamura, H. Sakiyama, S. G. Mayhew, Y. Asano, *Biochemistry* **2000**, *39*, 800-809.

[32] Y. Kato, T. Tsuda, Y. Asano, *Eur. J. Biochem.* **1999**, *263*, 662-670.

[33] Y. Kato, Y. Asano, *Protein Expression Purify.* **2003**, *28*, 131-139.

[34] Y. Kato, R. Ooi, Y. Asano, *Appl. Environ. Microbiol.* **2000**, *66*, 2290-2296.

[35] S. X. Xie, Y. Kato, H. Komeda, S. Yoshida, Y. Asano, *Biochemistry* **2003**, *42*, 12056-12066.

[36] T. Nagasawa, K. Ryuno, H. Yamada, *Biochem. Biophys. Res. Commun.* **1986**, *139*, 1305-1312.

[37] T. Nagasawa, H. Nanba, K. Ryuno, K. Takeuchi, H. Yamada, *Eur. J. Biochem.* **1987**, *162*, 691-698.

[38] T. Endo, I. Watanabe, *FEBS Lett.* **1989**, *243*, 61-64.

[39] T. Nagamune, H. Kurata, M. Hirata, J. Honda, H. Koike, M. Ikeuchi, Y. Inoue, A. Hirata, I. Endo, *Biochem. Biophys. Res. Commun.* **1990**, *168*, 437-442.

[40] T. Nagasawa, K. Takeuchi, H. Yamada, *Eur. J. Biochem.* **1991**, *196*, 581-589.

[41] M. S. Payne, S. Wu, R. D. Fallon., G. Tudor, S. B. Stieglitz, I. M. Jr. Turner, *Biochemistry* **1997**, *36*, 5447-5454.

[42] T. Yamaki, T. Oikawa, K. Ito, T. Nakamura, *J. Ferm. Bioeng.* **1997**, *83*, 474-477.

[43] R. A. Pereira, D. Graham, F. A. Rainey, D. A. Cowan, *Extremophiles* **1998**, *2*, 347-357.

[44] M. A. Wegman, U. Heinemann, A. Stolz, F. van Rantwijk, R. A. Sheldon, *Org. Process Res. Dev.* **2000**, *4*, 318-322.

[45] P. F. B. Brandao, S. Verseck, C. Syldatk, *Eng. Life Sci.* **2004**, *4*, 547-556.

[46] C. Ewert, S. Lutz-Wahl, L. Fischer, *Tetrahedron: Asymmetry* **2008**, *19*, 2573-2578.

[47] S. Y. Choi, Y. M. Goo, *Arch. Pharm. Res.* **1986**, *9*, 45-47.

[48] M. T. Reetz, K. Schimossek, *Chimia* **1996**, *50*, 668-669.

[49] Y. K. Choi, M. J. Kim, Y. Ahn, M. J. Kim, *Org. Lett.* **2001**, *25*, 4099-4101.

[50] D. Koszelewski, K. Tauber, K. Faber, W. Kroutil, *Trends Biotechnol.* **2010**, *28*, 324-332.

[51] M. Alexeeva, A. Enright, M. J. Dawson, M. Mahmoudian, N. J. Turner, *Angew. Chem. Int. Ed.* **2002**, *41*, 3177-3180.

[52] M. Alexeeva, R. Carr, N. J. Turner, *Org. Biomol. Chem.* **2003**, *1*, 4133-4137.

[53] H. Leisch, S. Grosse, H. Iwaki, Y. Hasegawa, P. C. K. Lau, *Can. J. Chem.* **2012**, *90*, 39-45.

[54] G. Li, J. Ren, H. Iwaki, D. Zhang, Y. Hasegawa, Q. Wu, J. Feng, P. C. K. Lau, D. Zhu,

Appl. Microbiol. Biotechnol. **2013**, DOI: 10.1007/s00253-013-5028-1

[55] D. Ghislieri, A. P. Green, M. Pontini, S. C. Willies, L. Rowles, A. Frank, G. Grogan, N. J. Turner, *J. Am. Chem. Soc.* **2013**, *135*, 10863–10869.

[56] D. Ghislieri, D. Houghton, A. P. Green, S. C. Willies, N. J. Turner, *ACS Catal*. **2013**, *3*, 2869-2872.

[57] M. Mure, S. A. Mills, J. P. Klinman, *Biochemistry* **2002**, *41*, 9269-9278.

[58] P. F. Fitzpatrick, Arch. *Biochem. Biophys*. **2010**, *493*, 13-25.

[59] C. Binda, A. Mattevi, D. E. Edmondson, *J. Biol. Chem*. **2002**, *277*, 23973-23976.

[60] P. Pietrangeli, R. Federico, B. Mondovi, L. Morpurgo, *J. Inorg. Biohem*. **2007**, *101*, 997-1004.

[61] V. Kohler, Y. M. Wilson, M. Durrenberger, D. Ghislieri, E. Churakova, T. Quinto, L. Knorr, D. Haussinger, F. Hollmann, N. J. Turner, T. R. Ward, *Nature Chem*. **2013**, *5*, 93-99.

[62] M. Durrenberger, T. Heinisch, Y. M. Wilson, T. Rossel, E. Nogueira, L. Knorr, A. Mutschler, K. Kersten, M. J. Zimbron, J. Pierron, T. Schirmer, T. R. Ward, *Angew. Chem. Int. Ed*. **2012**, *50*, 3026-3029.

[63] A. Hacisalihoglu, A. Jongejan, J. A. Jongejan, J. A. Duine, *J. Mol. Catal. B: Enzymatic.* **2000**, *11*, 81-88.

[64] T. Yajima, Y. Aizawa, M. Nishida, Y. Sakaguchi, T. Shiraiwa, *Biosci. Biotechnol. Biochem*. **2007**, *71*, 1338-1341.

[65] A. J. J. Straathof, J. A. Jongejan, *Enzyme Microb. Technol*. **1997**, *21*, 559-571.

[66] S. Klatte, R. M. Kroppenstedt, F. A. Rainey, *Syst. Appl. Microbiol.* **1994**, *17*, 355-360.

[67] M. Goodfellow, J. Chun, E. Stackebrandt, R. M. Kroppenstedt, *Int. J. Syst. Evol. Microbiol*. **2002**, *52*, 749-755.

[68] K. Bui, M. Maestracci, A. Thiery, A. Arnaud, P. Galzy, *J. Appl. Bacteriol.* **1984**, *57*, 183-190.

[69] L. Martinova, V. Kren, *Biocatal. Biotrans.* **2002**, *20*, 73-93.

[70] H. Saito and K. Miura, *Biochem. Biophis. Acta.* **1947**, *72*, 619-629.

[71] H. Ochman, A. S. Gerber, and D. L. Hrtl, *Genetics Society of America* **1988**, *120*, 621-623.

[72] Y. Kato, S.Yoshida, Y. Asano, *FEMS Microbiol. Lett.* **2005**, *246*, 243-249.

[73] R. Duran, M. Nishiyama, S. Horinouchi, T. Beppu, *Biosci. Biotechnol. Biochem.* **1993**, *57*, 1323-1328.

[74] S. H. Kim, P. Oriel, *Enzyme Microb. Technol.* **2000**, *27*, 492-501.

[75] K. Yasukawa, R. Hasemi, Y. Asano, *Adv. Synth. Catal.* **2011**, *353*, 2328-2332.

[76] E. G. Hibbert, F. Baganz, H. C. Hailes, J. M. Ward, G. J. Lye, J. M. Woodley, P. A. Dalby, *Biomol. Eng.* **2005**, 22, 11-19.

[77] S. Okazaki, A. Suzuki, T. Mizushima, T. Kawano, H. Komeda, Y. Asano, T. Yamane, *Biochemistry* **2009** *48*, 941-950.

[78] X. W. Liu, J. Ma, A. O. Colson, D. C. Doersen, F. H. Ebetino, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1223-1228.

[79] Y. Chu, Z. Shan, D. Liu, N. Sun, *J. Org. Chem.* **2006**, *71*, 3998-4001.

[80] Otwinowski, Z., and Minor, W., *Method Enzymol*, **1997**, *276*, 307-326.

[81] A. Vagin, A. Teplyakov, *J. Appl. Crystallogr.* **1997**, *30*, 1022-1025.

[82] M. D. Winn, *J. Synchrotron radiat.* **2003**, *10*, 23-25.

[83] P. Emsley, B. Lohkamp, W. G. Scott, K. Cowtan, *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *66*, 486-501.

[84] G. N. Murshudov, A. A. Vagin, E. J. Dodson, *Acta Crystallogr., Sect. D: Biol.*

Crystallogr. **1997**, *53*, 240-255.

[85] W. L. Delano, The PyMOL molecular graphics system, (Delano Scientific, S. C., CA., Ed.). **2002**.

[86] C. Setoyama, R. Miura, Y. Nishina, K. Shiga, H. Mizutani, I. Miyahara, K. Hirotsu, *J. Biochem*. **1996**, *119*, 1114-1117.

[87] V. I. Tishkov, S. V. Khoronenkova, *Translated from Biokhimiya* **2005**, *70*, 51-67.

[88] E. W. Hafner, D. Wellner, *Proc*. *Nat*l. *Acad*. *Sci*. **1971**, *68*, 987-991.

[89] J. W. Huh, K. Yokoigawa, N. Esaki, K. Soda, *J. Ferment. Bioeng.* **1992**, *74*, 189-190.

[90] T. M. Beard, N. J. Turner, *Chem*. *Commun*. **2002**, *38*, 246-247.

[91] F. R. Alexandre, D. P. Pantaleone, P. P. Taylor, I. G. Fotheringham, D. J. Ager, N. J. Turner, *Tetrahedron Lett*. **2002**, *43*, 707-710.

[92] A. Mattevi, M. A. Vanoni, F. Todone, M. Rizzi, A. Teplyakov, A. Coda, M. Bolognesi, B. Curti, *Proc*. *Natl*. *Acad*. *Sci*. **1996**, *93*, 7496-7501.

Publications

1. K. Yasukawa, R. Hasemi, Y. Asano, Dynamic kinetic resolution of α-aminonitrile to form chiral α-amino acid. *Adv. Synth. Catal.* **2011**, *353*, 2328-2332.

2. K. Yasukawa, Y. Asano, Enzymatic synthesis of chiral phenylalanine derivatives by a dynamic kinetic resolution of corresponding amide and nitrile substrates with multi enzyme system. *Adv. Synth. Catal.* **2012**, *354*, 3327-3332.

3. K. Yasukawa, S. Nakano, Y. Asano, Tailoring D-amino acid oxidase from pig kidney to *R*-stereoselective amine oxidase and its use in deracemization of α-methylbenzylamine. *Angew. Chem. Int. Ed.* **2014**, *53*, 4428-4431.