Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 32 (2005) 157–165
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Microbial deracemization of α-amino acids

Dai-Ichiro Kato, Kenji Miyamoto, Hiromichi Ohta*

Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan

Received 19 October 2004; received in revised form 2 December 2004; accepted 2 December 2004
Available online 29 December 2004

Abstract

We screened new microorganisms having deracemization activity of α-amino acids and isolated some active strains. Whole cells of these strains were capable of inverting the chirality of 4-chlorophenylalanine from D- to L-configuration. In particular, *Nocardia diaphanozonaria* JCM 3208 exhibits the deracemization activity towards wide variety of α-amino acids, such as phenylglycine and 2-aminoheptanoic acid. Examination of the time course of the reaction revealed that α-keto acid was produced as the key intermediate. In addition, mechanistic studies using cell-free extract suggested that deracemization process is realized by two enzymatic reactions; D-stereoselective oxidative deamination reaction and L-selective transamination reaction. Finally, we could establish the reaction conditions utilizing the cell-free system to proceed the deracemization of phenylalanine, which was degraded when the whole cells were used as the biocatalyst.

Keywords: α-Amino acids; D-Phenylalanine degrading microorganisms; Enantioselective inversion of configuration; Microbial deracemization

1. Introduction

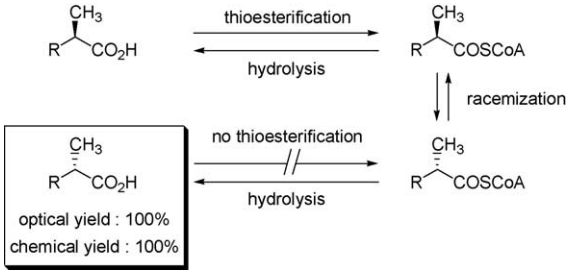
The preparation of optically active compounds has been attracting much attention because chirality is essentially important for the physiologically active compounds. There have been known a variety of chemical and enzymatic methods for this purpose. Among them, deracemization reaction is an unique and promising biotransformation due to its novelties and environmental friendliness, because this process gives optically active compounds starting from the corresponding racemates without changing the chemical structures. Thus, in theory, enantiomerically pure compounds are obtained in 100% atom efficiency. Although, the term "deracemization" is not so familiar at present, this process has become attracting attention in the biotransformation filed through some pioneering works [1]. Recently, we reported the deracemization reaction of α-methyl carboxylic acids using an actinomyces *Nocardia diaphanozonaria* JCM 3208 [2]. This process has been estimated to involve a racemization reaction of an intermediate, which was derived from one enantiomer. Thus the biotransformation can be said as "enantioselective racemization" as a whole (Scheme 1).

In this paper, we would like to report another trick, i.e., enantioselective inversion of configuration. In the present study, to widen the usefulness of deracemization technique, we have screened and isolated many kinds of novel microorganisms having the phenylalanine deracemization activity. These strains were capable of deracemizing the wide variety of other α-amino acids. Because of the novelty of this bioconversion, we could access little information about tracing the history of α-amino acid deracemization. About 40 years ago, Chibata et al. reported the enantioselective isomerization reaction of phenylalanine [3]. In 2000, Hasegawa et al. described metabolic stereoselective chiral inversion of leucine in vivo system of rat [4]. In both reports, the authors suggested a two-step reaction mechanism via an intermediate, α-keto acid, to achieve the deracemization (Scheme 2).

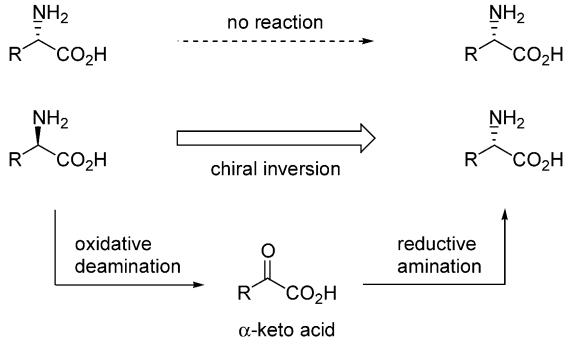
It is an interesting question whether the strains isolated in our study also realize the deracemization by the same reaction pathways. From the investigation using whole cells and cell-free extract, we could clarify that the present deracem-

* Corresponding author. Tel.: +81 45 566 1703; fax: +81 45 566 1551.
E-mail address: hohta@bio.keio.ac.jp (H. Ohta).

1381-1177/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2004.12.002



Scheme 1. Enantioselective racemization to realize the deracemization reaction.



Scheme 2. Enantioselective inversion of configuration to realize the deracemization reaction.

ization was also achieved via the combination of two enzymatic reactions, i.e., D-selective deamination and L-selective transamination. Moreover, we have succeeded to establish the reaction conditions under which the deracemization reaction of phenylalanine and phenylglycine proceeded in high efficiency by the addition of artificial cofactors to the cell-free systems.

2. Materials and methods

2.1. Materials and microorganisms

4-Chlorophenylpyruvic acid [5] and 2-oxo-3-methylbutanoic acid [6] were prepared according to the procedure described in literatures. All other chemicals were commercially available and used without further purification. L-Phenylalanine dehydrogenase (L-PheDH, origin; *Sporosarcina* sp.) was purchased from Wako Pure Chemical Industries, Ltd.

The microorganisms which were used in this experiment were as follows: *N. diaphanozonaria* JCM 3208, *Sinorhizobium meliloti* NBRC 14782 or ATCC 51124, *Mesorhizobium loti* MAFF 303099, *Streptmyces roseus* NBRC 12818, *Streptomyces coelicolor* IAM 1023, *Pseudomonas chlororaphis* NBRC 3521, *Pseudomonas oxalaticus* NBRC 13593, and *Pseudomonas* sp. KU 2071. *Pseudomonas* sp. KU 2071 was isolated from soil, which was numbered as soil isolate No. 9 (Table 1, entry 9).

2.2. Analytical

The concentration of the α-amino acid in the reaction mixture was measured by TNBS method [7] or HPLC, which performed with a COSMOSIL 5C18-ARII (150 mm × 4.6 mm) column at room temperature, and the compounds were detected at 254 nm. Separation was achieved employing an isocratic mobile phase consisting H_2O/acetonitrile (20/1) at a flow rate of 0.5 mL/min. Under these conditions, retention times of phenylglycine and phenylalanine were 6.9 and 13.9 min, respectively. The concentration of α-keto acid was determined by derivation to 2,4-dinitrophenylhydrozone [8]. Benzoylformic acid, which was formed by the deamination reaction of phenylglycine, was also detected on HPLC, which was performed with the above-mentioned COSMOSIL 5C18-ARII column. Separation condition was the same ex-

Table 1
Deracemization of α-amino acids

Entry	Strain	ee (%)			
		4-Cl-Phe[a]	Phenylglycine	C7[b]	*Tert*-Leu[c]
1	*N. diaphanozonaria* JCM 3208[d]	>99	>99	97	75
2	*S. meliloti* NBRC 14782[d]	>99	N.P.[e]	28	N.P.[e]
3	*S. meliloti* ATCC 51124[d]	98	>99	–	–
4	*M. loti* MAFF 303099[f]	94	42	–	–
5	*S. roseus* NBRC 12818[d]	95	38	56	N.P.
6	*S. coelicolor* IAM 1023[d]	70	N.P.	–	–
7	*P. chlororaphis* NBRC 3521[d]	99	–	–	–
8	*P. oxalaticus* NBRC 13593[d]	99	–	–	–
9	*Pseudomonas* sp. KU 2071 (soil isolate No. 9)[g]	95	>99	–	–

[a] 4-Chlorophenylalanine.
[b] 2-Aminoheptanoic acid.
[c] *Tert*-leucine.
[d] The wet cells from 10 mL medium were resuspended in 10 mL KPB containing 1 mg/mL racemic substrate and incubated with shaking for 48 h at 30 °C.
[e] The deracemization reaction did not proceed.
[f] The wet cells from 100 mL medium were resuspended in 10 mL KPB containing 1 mg/mL racemic substrate and incubated with shaking for 144 h at 30 °C.
[g] The wet cells from 10 mL medium were resuspended in 5 mL KPB containing 1 mg/mL racemic substrate and incubated with shaking for 24 h at 30 °C.

cept for the mobile phase, which was H_2O/acetonitrile/TFA (2/1/0.1%). Under these conditions, phenylglycine and benzoylformic acids were eluted in 6.8 and 9.5 min, respectively. The enantiomeric excess (ee) of the product was determined by a Chiralpack WM (250 mm × 4.6 mm) column or a Crownpack CR(+) (150 mm × 4.0 mm) column. Protein concentration was determined by the method of Bradford [9] with the protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and bovine serum albumin (BSA) was used as the standard.

2.3. Screening of microorganisms

Microbial strains were obtained from the glycerol stock cells in our laboratory or isolated from soil in Japan. Soil microorganisms were screened in minimal D-phenylalanine medium, which consisted of glycerol (0.2 g), $(NH_4)_2HPO_4$ (5 g), D-phenylalanine (5 g), yeast extract (0.2 g), Na_2HPO_4 (10 g), K_2HPO_4 (2 g), $MgSO_4{\cdot}7H_2O$ (0.3 g), $FeSO_4{\cdot}7H_2O$ (10 mg), $ZnSO_4{\cdot}7H_2O$ (8 mg), $MnSO_4{\cdot}7H_2O$ (8 mg) and 1000 mL of distilled water (pH 7.2). Soil samples were added to this medium (10 mL) and the mixture was shaken for 7 days at 30 °C. This broth (100 μL) was added to the fresh medium (10 mL) and cultured again for 7 days. After three times repetition of this operation, the broth was spread on an agar plate consisting of the same components. The colonies grown were picked up and assayed for the deracemization activity. The activity of each strain was measured in two-step assay, i.e., plate color assay followed by HPLC assay. Identification of soil isolate No. 9 was carried out at NCIMB Japan Co. Ltd. (Shimizu, Japan). The strain No. 9 was gram-negative, formed rods, non-spore-forming, and without flagella. It produces no acid and gas from glucose. Both catalase and oxidase reaction were found to be positive. Type of 16S rDNA showed that soil isolate No. 9 is closely related to *Pseudomonas nitroreducens* (99.6% homology) and *Pseudomonas pseudoalcaligenes* spp. *pseudoalcaligenes* (98.1%). Based on these results, the soil isolate No. 9 was identified as *Pseudomonas* sp. and registered to our stock culture as KU 2071.

2.4. Deracemization activity assay

2.4.1. Plate color assay using whole cell system

Deracemization activity was detected by the generation of L-phenylalanine from D-isomer utilizing the enantioselective deamination reaction catalyzed by L-phenylalanine dehydrogenase (L-PheDH). Streaked cells on an agar plate were transferred to the Hybond-N^+ membrane. After incubation for several hours at 30 °C, membrane was treated with 5 mL of a solution of lysozyme hydrochloride (20 mg/mL) in 50 mM potassium phosphate buffer (KPB, pH 7.0) containing 1 mM EDTA and incubated for 2 h at 37 °C. The cells were lysed completely. This membrane was transferred to an assay plate (20 mM D-phenylalanine, 5 mM NAD^+, 0.3 mg/mL nitrobluetetrazolium chloride, 0.05 mg/mL phenazine metasulfate and 1.7% agar), to which 0.5 unit of L-PheDH was applied before use, and incubated for 2 h at 30 °C. When the enzyme system of the original cells converted D-phenylalanine to its L-isomer, then the sample turned blue due to the oxidation of L-phenylalanine to phenylpyruvic acid.

2.4.2. HPLC assay

Each isolated strain was cultured with shaking in 10 mL of the medium, which was used before for the growth of *N. diaphanozonaria* [2] for 48 h at 30 °C. The cells were harvested and washed with 100 mM KPB (pH 7.0). This wet cells were resuspended in 5 mL of KPB containing 1 mg/mL of α-amino acid. After shaking for 24 h at 30 °C, the ee of the product was determined by HPLC.

2.5. Preparation of the cell-free extract

The microorganism was cultured with shaking in 10 mL of the above-mentioned *N. diaphanozonaria* medium [2] for 48 h at 30 °C. This pre-cultured cells were then added to 90 mL of the fresh medium, and the mixture was shaken for further 24 h. The cells were harvested and washed with 100 mM KPB (pH 7.0). These wet cells were resuspended in 10 mL of ice-cooled 100 mM MOPS-NaOH buffer (pH 7.25) containing 1 mM DTT. The cells were disrupted by French press. The cell debris were removed by centrifugation (15,000 rpm, 10 min), and dialyzed in MOPS-NaOH buffer overnight. This cell-free extract was used without further purification for the mechanistic investigation of deracemization reaction. Protein concentration was 4.8 mg/mL (*S. meliloti* ATCC 51124) and 3.2 mg/mL (*Pseudomonas* sp. KU 2071).

2.6. Enzymatic assay

2.6.1. D-Amino acid deaminating activity assay using the cell-free extract

The activity of D-amino acid oxidation was measured spectrophotometrically by the reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm in the presence of *N*-methylphenazonium methosulfate (PMS) according to the report by Tsuchiya et al. [10]. The assay mixture contained 50 mM KPB (pH 7.0), 2.4 mM D- or L-phenylalanine, 0.24 mM DCIP, 0.24 mM PMS and 50 μL of the cell-free extract in a total volume of 1 mL.

2.6.2. Aminotransferase activity assay using the cell-free extract

The activity of aminotransferase was determined by the conversion of α-keto acid to the corresponding α-amino acid. The assay mixture containing 1.2 mM α-keto acid, 6.7 mM amino donor (for example, L-glutamic acid), 0.6 mM PLP, and 100 μL of the cell-free extract in a total volume of 1 mL of 100 mM Tris–HCl buffer (pH 8.5) was incubated for 10 h at 30 °C. The α-amino acid resulting from the α-keto acid was determined by HPLC.

2.6.3. Microbial deracemization of α-amino acids using the cell-free extract

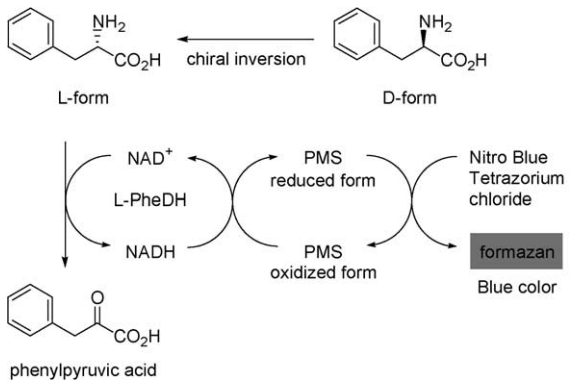
The reaction conditions of deracemization of phenylalanine were as follows. A mixture of 1.2 mM D-phenylalanine or DL-4-chlorophenyalanine, 0.24 mM DCIP, 0.24 mM PMS, 6.7 mM L-glutamic acid, 0.6 mM PLP, and 100 μL of the cell-free extract in a total volume of 1 mL of 100 mM Tris–HCl buffer (pH 8.5) was incubated at 30 °C under the light-shielded condition. After the incubation for appropriate time, the ee of the product was determined by HPLC.

3. Results and discussion

3.1. Screening for D-phenylalanine degrading microorganisms

Microbial strains capable of utilizing D-phenylalanine as the source of carbon were isolated by enrichment culture in minimal D-phenylalanine medium. The possibilities of D-phenylalanine deracemizing activity of these strains were tested via two steps. First examination was a plate color assay. The cell lysete of microorganism was applied on a plate containing D-phenylalanine and L-PheDH. If the enzyme catalyzed the formation of L-phenylalanine from its D-form, the resulting L-form is oxidized to phenylpyruvic acid with simultaneous formation of NADH, which is coupled with the change of the color of the reaction mixture to blue as illustrated in Scheme 3.

In this assay system, however, strains having the racemization activity for phenylalanine were also picked up. To remove these strains, we have carried out the second examination to measure the ee of the product directly starting from enantiomerically pure D- or L-phenylalanine and DL-4-chlorophenylalanine. Because, phenylalanine was degraded gradually when it is subjected to the reaction with whole cells, we measured the ee at the early stage of the incubation and screened the strains, which have the ability of converting the chirality of D-phenylalanine to L-form. On the other hand, 4-chlorophenylalanine was stable under these conditions, and thus clear results were obtained. Thus, we have tried about 50 strains (bacteria, fungi, and actinomycetes). Among them, nine kinds of strains were chosen for further investigation of substrate specificity. These strains exhibited the deracemization activity regardless to the induction by D-phenylalanine. It may be worthy to mention that although we have also screened microorganisms having the deracemization activity toward L-phenylalanine via the same procedure as in the case of aiming at D-phenylalanine, no strains were found so far.



Scheme 3. Concept of plate color assay for the screening of phenylalanine deracemizing microorganisms.

3.2. Substrate specificity of D–phenylalanine degrading bacteria

One of the most powerful deracemization activity was detected in an actinomyces *N. diaphanozonaria* (Table 1, entry 1). We have already reported that this strain has a deracemization ability toward various kinds of α-methyl carboxylic acids, such as 2-phenylpropanoic acid, 2-aryloxypropanoic acid, and 2-methyl-3-phenylpropanoic acid [2]. On the other hand, a kind of fungi *Cordyceps militaris* ATCC 341634, which is known as exhibiting the deracemization activity for 2-methyl carboxylic acids [11], could not invert the chirality of D-phenylalanine and exhibited only the D-isomer-degradation activity. To the best of our knowledge, *N. diaphanozonaria* is the first example of a microorganism, which is capable of deracemizing the two types of substrates, α-methyl carboxylic acids and α-amino acids. When phenylalanine was subjected to the whole cells of *N. diaphanozonaria*, they were rapidly degraded. On the other hand, non-natural α-amino acid such as 4-chlorophenylalanine and phenylglycine were added to the whole cell system of this microorganism, no degradation was observed and L-enantiomers of high ee were recovered in almost quantitative yield. This strain exhibits the deracemization activity for wide variety of α-amino acids, not only 2-aminoheptanoic acid with a medium chain-length alkyl substituent, but also *tert*-leucine, which has a bulky *tert*-butyl substituent at β-position. It is interesting that cyclic amino acids, such as proline and indoline-2-carboxylic acid, were not affected by *N. diaphanozonaria*. Other microorganisms could also deracemize α-amino acids as shown in Table 1. Although, previously reported phenylalanine deracemizing microorganisms were a kind of bacterium and actinomycetes, such as *Pseudomonas* sp. [3], we have discovered new types of microorganisms *S. meliloti* (entries 2, 3) and *M. loti* (entry 4). These strains were a group of the nitrogen-fixing symbiotic bacteria and exhibited the high deracemization activities toward phenylalanine derivatives as well. It is interesting that while the strain ATCC 51124 (entry 3) had the deracemization activity toward D- or DL-phenylglycine, the strain NBRC 14782 (entry 2) dose not accept this compound. Moreover, almost all the soil strains have deracemization activity only toward phenylalanine derivatives, *Pseudomonas* sp. (entry 9) alone could accept not only phenylalanine but also phenylglycine as the substrate.

3.3. Mechanistic investigation using the whole cell system

From the early studies, it is supposed that microbial deracemization of α-amino acid was realized by the combination of two enzymatic reactions, D-selective deamination followed by L-selective amination [3,4]. If this supposition is true, α-keto acid will be formed as the key intermediate. Thus, we tried to detect this intermediate using the whole cell system of three kinds of strains, *N. diaphanozonaria*, *S. meliloti*, and *Pseudomonas* sp. First, the time courses of the change of concentrations of compounds in the reaction mixture were measured. However, when the starting material is DL-phenylalanine, the rapid degradation proceeded and no phenylpyruvic acid was detected. On the other hand, in case of 4-chlorophenylalanine, smart results were obtained and the chiral inversion process from D- to L-configuration was clearly visualized (Fig. 1). Moreover, the corresponding α-keto acid was detected in the whole cell system of *S. meliloti* and *Pseudomonas* sp., although we failed to detect the same intermediate in the case of *N. diaphanozonaria*. In the case that the substrate was DL-phenylglycine, the reaction profile for *N. diaphanozonaria* and *Pseudomonas* sp. were similar to those of 4-chlorophenylalanine (Fig. 2). On the other hand, *S. meliloti* showed no activity to this compound (data are not shown).

Next, to clarify the reaction mechanism, 4-chlorophenylpyruvic acid was added to the whole cell systems. All three strains have demonstrated the ability of converting the α-keto acid to L-4-chlorophenylalanine, and no D-4-chlorophenylalanine was detected in the reaction mixture (Fig. 3). Among these three strains, *N. diaphanozonaria* have the highest ability for the amino acid production. Although the corresponding α-keto acid was not detected in the reaction mixture in the case of *N. diaphanozonaria*, probably because of this high amination activity, it is clear that the corresponding α-keto acid is the important intermediate of chiral inversion process. Thus the deracemization reaction is considered to be realized by the combination of two enzymatic reactions.

3.4. Cofactor requirement in the cell-free systems

From the mechanistic investigation using the whole cell systems, α-keto acid is supposed to be an important intermediate in the course of the chiral inversion process. Thus

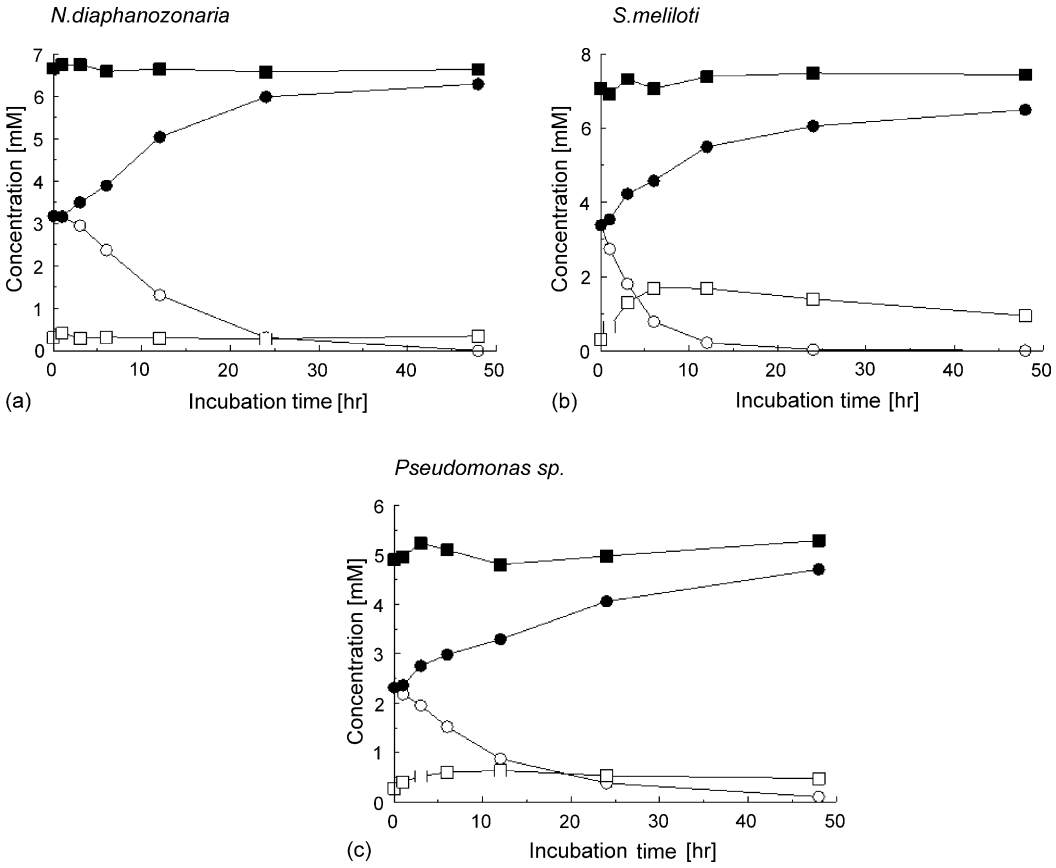


Fig. 1. The change of 4-chlorophenylalanine concentration in the reaction mixture. (a) *N. diaphanozonaria* JCM 3208; (b) *S. meliloti* NBRC 14782; (c) *Pseudomonas* sp. KU 2071. The cells harvested from 100 mL medium were resuspended in 100 mL of 100 mM potassium phosphate buffer with the substrate (1 mg/mL) in a 500 mL shaking culture (Sakaguchi) flask. The flask was shaken at 30 °C. Symbols: (◯) D-4-chlorophenylalanine; (●) L-4-chlorophenylalanine; (□) 4-chlorophenylpyruvic acid; (■) total concentration of above three compounds.

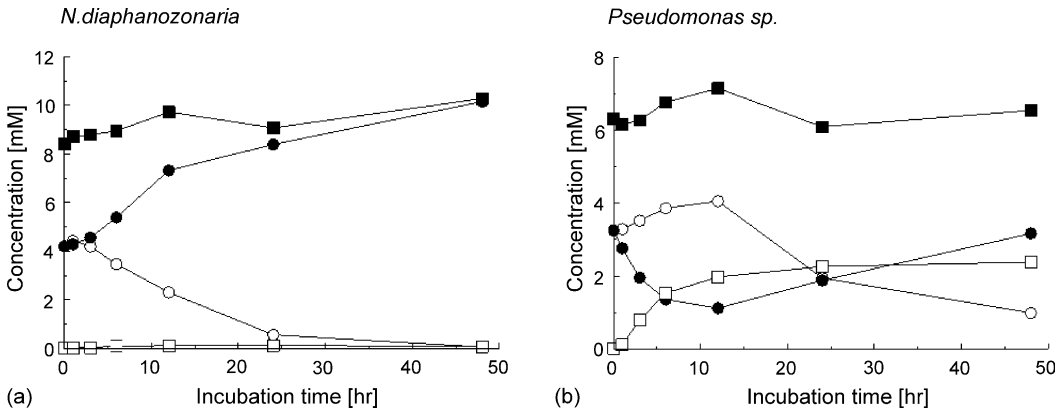


Fig. 2. The change of phenylglycine concentration in the reaction mixture. (a) *N. diaphanozonaria* JCM 3208; (b) *Pseudomonas* sp. KU 2071. The cells harvested from 100 mL medium were resuspended in 100 mL of 100 mM potassium phosphate buffer with the substrate (1 mg/mL) in a 500 mL shaking culture (Sakaguchi) flask. The flask was shaken at 30 °C. Symbols: (◯) D-phenylglycine; (●) L-phenylglycine; (□) 4-benzoylformic acid; (■) total concentration of above three compounds.

the deracemization is considered to proceed via two enzymatic reactions and the most probable possible pathway is the combination of amino acid oxidation and transamination or reductive amination. To elucidate this biotransformation system more clearly, we have examined the two enzymatic activities separately using cell-free extract. In the case of *N. diaphanozonaria*, however, no deracemization activity could be detected in the cell-free extract probably because of the instability of the enzyme system. On the other hand, cell-free extract of *S. meliloti* and *Pseudomonas* sp. kept the deracemization activity after centrifugation of cell disruption by French press. When this crude mixture was dialyzed, how-

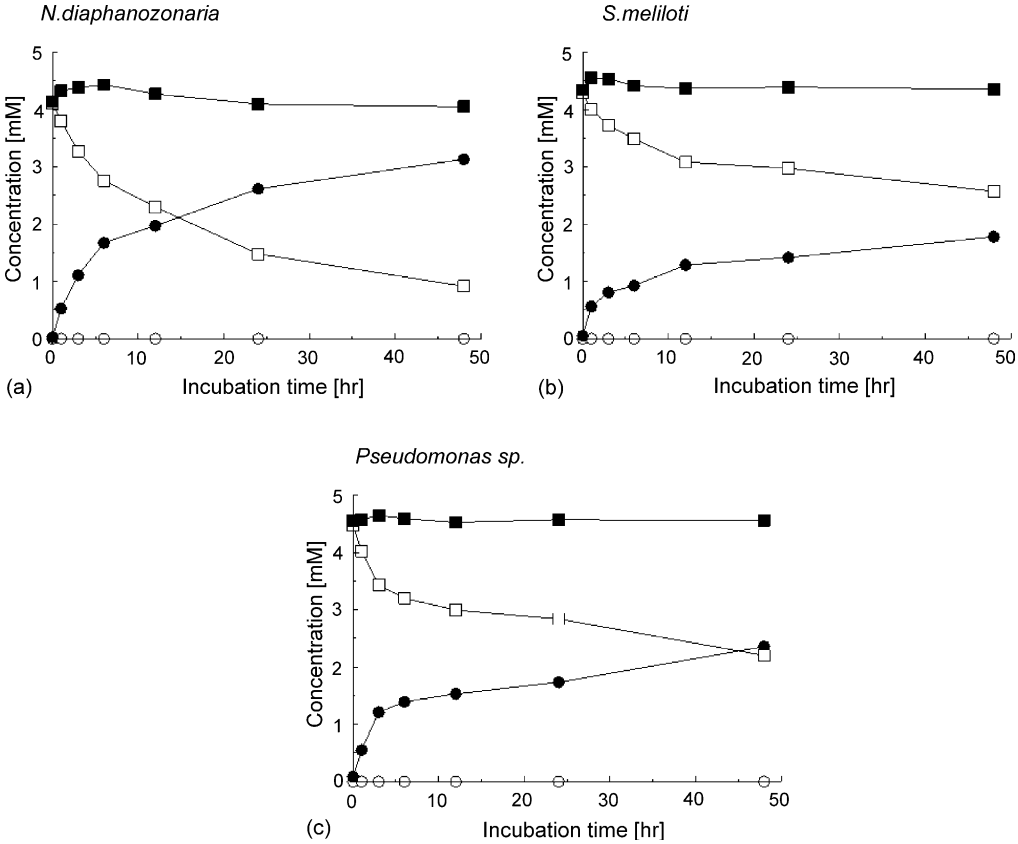


Fig. 3. Formation of 4-chlorophenylalanine from 4-chlorophenylpyruvic acid using the whole cell system. (a) *N. diaphanozonaria* JCM 3208; (b) *S. meliloti* NBRC 14782; (c) *Pseudomonas* sp. KU 2071. The cells harvested from 100 mL medium were resuspended in 100 mL of 100 mM potassium phosphate buffer with the substrate (1 mg/mL) in a 500 mL shaking culture (Sakaguchi) flask. The flask was shaken at 30 °C. Symbols: (◯) D-4-chlorophenylalanine; (●) L-4-chlorophenylalanine; (□) 4-chlorophenylpyruvic acid; (■) total concentration of above three compounds.

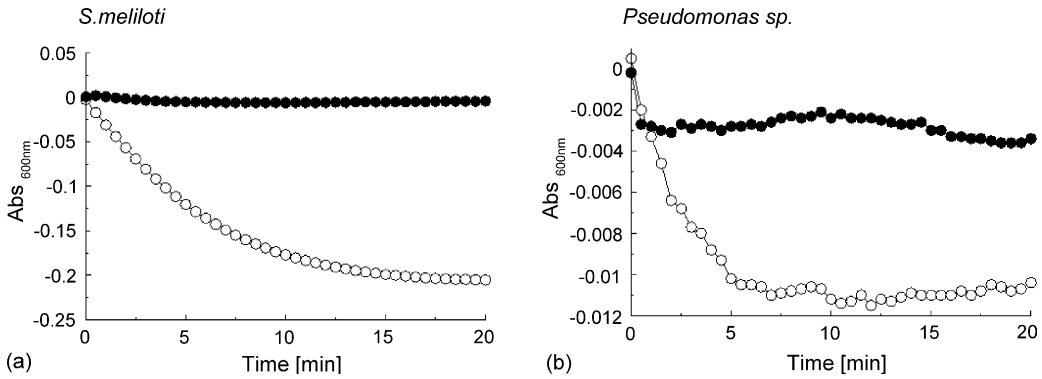


Fig. 4. The amino acid oxidizing activity of the cell-free systems measured by DCIP method. (a) *S. meliloti* ATCC 51124; (b) *Pseudomonas* sp. KU 2071. Symbols: (◯) D-phenylalanine; (●) L-phenylalanine.

ever, deracemization activity decreased dramatically, which suggests that this deracemization process needs some cofactors at least for either one step of the two.

3.5. Phenylalanine oxidation activity

To investigate the cofactor requirement of the oxidizing enzyme system, we studied the effect of the reaction atmosphere using the resting cell system. Even when the reaction was performed under argon, deracemization reaction proceeded smoothly and ee of the product exhibited almost the same value with those obtained under the aerobic conditions (Table 2). The same results were obtained using the cell-free system of *S. meliloti*. Thus the oxidizing system was revealed to be O_2 independent.

As described above, the enzyme activity of deracemizing phenylalanine reduced dramatically, when the dialyzed cell-free extract was used and the electron acceptor of the oxidation step was not O_2. Thus there should be some electron acceptor in the cell-free extract. In many cases, artificial electron acceptors could be used for the regeneration of the cofactor coupled with oxidizing enzymes such as dehydrogenase. We tried PMS as an electron acceptor and detected the reduction of this artificial cofactor by coupling with DCIP at 600 nm. As shown in Fig. 4, D-selective oxidation was observed in the dialyzed cell-free extract of *S. meliloti* and *Pseudomonas* sp., the former having the stronger activity. In the absence of PMS, no reduction of DCIP was observed.

Table 2
Effect of reaction atmosphere on the deracemization of DL-4-chlorophenylalanine

Entry	Strain	ee (%)	
		Under aerobic	Under Ar
1	*N. diaphanozonaria* JCM 3208	98	>99
2	*S. meliloti* NBRC 14782	87	87
3	*Pseudomonas* sp. KU 2071	91	90

The wet cells from 10 mL broth were resuspended in 10 mL KPB containing 1 mg/mL racemic 4-chlorophenylalanine and incubated with shaking for 24 h at 30 °C under Ar or aerobic conditions.

3.6. Phenylalanine-forming activity from phenylpyruvic acid

The activity of oxidizing D-phenylalanine was confirmed by the above experiments. Next, the formation of phenylalanine from phenylpyruvic acid was examined. There are two major possibilities for the formation of phenylalanine from phenylpyruvic acid, i.e., transamination reaction and reductive amination reaction. Actually, the L-phenylalanine production activity was detected in the cell-free extract of *S. meliloti* and *Pseudomonas* sp. in the presence of PLP and L-glutamic acid. Moreover, L-selective transamination reaction of phenylalanine to phenylpyruvic acid was observed on the addition of PLP and α-ketoglutaric acid to the cell-free extract. In the case of *Pseudomonas* sp., we examined transamination reaction using benzoylformic acid as the acceptor. The transamination activity increased or at least showed the same level as that of L-glutamic acid by the addition of hydrophobic amino acids, such as L-valine (167%), L-leucine (135%), L-methionine (81%), L-tryptophan (79%), and L-phenylalanine (78%). On the other hand, in the reductive amination system using NH_4Cl or urea with NADH or NADPH, no formation of phenylalanine was observed. Thus, it can be concluded that the second step of deracemization process is transamination reaction to α-keto acids.

Table 3
Deracemization reaction using cell-free system of *S. meliloti* ATCC 51124

Entry	Substrate	Time (h)	pH	EA[a]	ee (%)
1	D-Phe	11	7.0	DCIP, PMS	77 (D)
2	D-Phe	66	7.0	DCIP, PMS	60 (D)
3	D-Phe	38	8.5	DCIP, PMS	95 (L)
4	D-Phe	38	8.5	PMS	95 (L)
5	DL-4-Cl-Phe	38	8.5	DCIP, PMS	99 (L)
6	DL-4-Cl-Phe	38	8.5	PMS	99 (L)
7	D-phenylglycine	23	8.5	PMS, DCIP	29 (L)
8	D-Phenylglycine	54	8.5	PMS, DCIP	71 (L)
9	D-Phenylglycine	95	8.5	PMS, DCIP	81 (L)

[a] Artificial electron acceptor.

Table 4
The cofactor requirement experiment using the cell-free system of *S. meliloti* ATCC 51124

Entry	Cofactors	D-Phe		DL-4-Cl-Phe		D-phenylglycine	
		Yield (%)	Ee (%)	Yield (%)	ee (%)	Yield (%)	ee (%)
1	All[a]	100	96 (L)	100	>99 (L)	100	29 (L)
2	For transaminase[b]	100	7 (D)	98	76 (L)	100	21 (D)
3	For oxidase[c]	14	72 (L)	51	>99 (L)	45	42 (D)
4	None[d]	75	82 (D)	72	56 (L)	81	79 (D)

[a] DCIP, PMS, PLP, L-Glu.
[b] PLP, L-Glu.
[c] DCIP, PMS.
[d] No cofactors added.

3.7. The estimation of reaction mechanism

So far, the reaction mechanism can be summarized as follows. These strains formed α-keto acid as the intermediate during the chiral inversion process via oxidative deamination reaction. This step is enantioselective, only the D-form being converted to the corresponding α-keto acid. In addition, deamination reaction was catalyzed by O_2-independent oxidizing enzyme, which accepted PMS as an electron acceptor. Second step of the chiral inversion process was catalyzed by aminotransferase. This enzyme worked in the presence of PLP and L-glutamic acid, and transformed the α-keto acid to the corresponding L-amino acid. By the combination of these two enzymatic reactions, deracemization of α-amino acids is realized as a whole.

3.8. The optimal conditions for deracemization reaction by the cell-free extract of S. meliloti

Finally, we examined the optimal reaction conditions to proceed the deracemization reaction in the cell-free systems of *S. meliloti*. As expected, under the light-shielded condition, D-phenylalanine and DL-4-chlorophenylalanine were inverted to the L-form in the cell-free extract of *S. meliloti* in the presence of PMS, DCIP, PLP, and L-glutamic acid (Table 3, entries 3 and 5).

In this system, however, no chiral inversion proceeded when the reaction was conducted under light, probably due to the instability of PMS toward light. The pH of the reaction medium was also important, and the highest activity was observed at pH 8.5. The rate of reaction greatly decreased under pH 7.0 (entries 1 and 2). The addition of DCIP is not necessarily required for the reaction, as the rate of reactions was not affected by the presence or absence of the reagent (entries 3 and 4, 5 and 6). The deracemization reaction of phenylglycine was also realized under the same conditions as those for phenylalanine deracemization, although, the rate of reaction was slower compared to the case of phenylalanine, and the complete deracemization was not achieved even after 95 h incubation (entries 7–9).

As to the cofactor requirement, it became clear that the deracemization proceeded only in the presence of both cofactors for deamination and transamination (Table 4, entry 1). When the cofactors were not added in the assay system, the deracemizing activity could be detected only slightly (entry 4). On the other hand, in the presence of PLP and glutamic acid (cofactors for transaminase), the deracemization reaction proceeded more efficiently as shown in entry 2. In the case that DL-4-chlorophenylalanine was employed as the substrate, the ee of the resulting L-form was reduced to 70%. The effect of the absence of cofactors for transaminase was clear as shown in entry 3. In this case, the yield of α-amino acid was lower than the case of other. This is because of the accumulation of α-keto acids due to the inhibition of reductive amination process owing to the lack of the cofactors for this step.

4. Conclusion

We have screened and isolated some microorganisms having the activity of phenylalanine deracemization. All strains have the ability to invert the chirality of D- to L-configuration and no microorganism was isolated having the opposite selectivity. Among them, *N. diaphanozonaria* JCM 3208 exhibited the most strong deracemization activity and accepted wide variety of α-amino acids as substrates. Other strains also exhibited the deracemization activity for various kinds of α-amino acids. From the mechanistic investigation using the whole cells and cell-free extract, we have clarified that chiral inversion process was achieved by the combination of two enzymatic reactions. First step is catalyzed by D-amino acid deaminating enzyme, which worked under the cell-free systems in the presence of artificial electron acceptor. Second step is transaminase catalyzed L-amino acid production by the aid of PLP and L-glutamic acid. Finally, we have established the reaction conditions to proceed the deracemization reaction of phenylalanine and phenylglycine in high efficiency by the addition of artificial cofactors in the cell-free systems.

Acknowledgements

DK acknowledges to the financial support by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (JSPS Research Fellowships for

Young Scientists). This study was performed through Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

References

[1] N.J. Turner, Curr. Opin. Chem. Biol. 8 (2004) 114.
[2] D.-I. Kato, K. Miyamoto, H. Ohta, Tetrahedron: Asymmetr. 15 (2004) 2965.
[3] I. Chibata, T. Tosa, R. Sano, Appl. Microbiol. 13 (1965) 618.
[4] H. Hasegawa, T. Matsukawa, Y. Shinohara, T. Hashimoto, Drug Metab. Dispos. 28 (2000) 920.
[5] J. Tao, K. McGee, Org. Proc. Res. Dev. 6 (2002) 520.
[6] X. Creary, J. Org. Chem. 52 (1987) 5026.
[7] R. Fields, Meth. Enzymol. 25 (1972) 464.
[8] V. Job, G.L. Marcone, M.S. Pilone, L. Pollegioni, J. Biol. Chem. 277 (2002) 6985.
[9] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[10] S. Tsuchiya, K. Miyamoto, H. Ohta, Biotechnol. Lett. 14 (1992) 1137.
[11] W. Rhys-Williams, M.J. Thomason, Y.-F. Hung, G.W. Hanolon, A.W. Lloyd, Chirality 10 (1998) 528.