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FULL PAPER

Chemoenzymatic method for enantioselective synthesis of (*R*)-2-phenylglycine and (*R*)-2-phenylglycine amide from benzaldehyde and KCN using difference of enzyme affinity to the enantiomers

Nobuhiro Kawahara[a,b], Yasuhisa Asano*[a,b]

Dedication ((optional))

Abstract: In general, enzymatic and chemoenzymatic methods for asymmetric synthesis of α-amino acids are performed using highly enantioselective enzymes. The enzymatic reactions using α-aminonitrile as a starting material have been performed using reaction conditions apart from the chemical Strecker synthesis. We developed a new chemoenzymatic method for the asymmetric synthesis of α-amino acids from aldehydes and KCN by performing Strecker synthesis and nitrilase reaction in the same reaction mixture. Nitrilase AY487533 that showed rather low enantioselectivity in hydrolysis of 2-phenylglycinonitrile (2PGN) to 2-phenylglycine (2PG) was utilized in the hydrolysis of aminonitrile formed from benzaldehyde and KCN *via* 2PGN by Strecker synthesis, preferentially synthesizing (*R*)-2PG with more than 95% yield and enantiomeric excess (ee). The method was also utilized for the synthesis of (*R*)-2-phenylglycine amide ((*R*)-2PGNH$_2$) from benzaldehyde and KCN by the chemoenzymatic reaction in the presence of a mutated nitrilase AY487533 W186A, which catalyzes the conversion of 2PGN to 2PGNH$_2$.

Introduction

Optically active α-amino acids are versatile intermediates for the production of various pharmaceuticals and fine chemicals. Hence, simple methods for production of chiral amino acids are required. Now, chiral α-amino acids are widely synthesized from aldehydes or ketones by Strecker synthesis and chemical hydrolysis of α-aminonitriles followed by the kinetic resolution of the resulting racemic amino acids or their derviatives. [1] However, the chemical methods are not always adequate for enantioselective production of (*R*)- or (*S*)-□α-amino acids, because asymmetric Strecker synthesis is quite limited by the preparation of *N*-substituted aminonitriles. [2] The methods have drawbacks such as the requirement of special chiral catalysts or expensive chiral auxiliaries that are difficult to obtain. Furthermore, the chemical methods require a harsh pH condition and high temperature to hydrolyze the α-aminonitrile and remove the *N*-substituent. [3] So far, an industrially effective method of chiral amino acid synthesis is unavailable.

To improve the chemical methods, nitrile-metabolizing enzymes such as nitrilases and nitrile hydratases have been developed, because the stereoselective hydrolysis of aminonitriles by enzymes is a simple approach for the synthesis of amino acids or amino acid amides. [4] Thus, nitrilases, which catalyze the conversion of nitriles into carboxylic acids, are utilized for stereoselective production of α-amino acids from racemic α-aminonitriles; [5] For example, optically pure α-amino acids such as (*S*)-alanine [6] (*S*)-leucine [7] and (*S*)-phenylglycine [8] were produced by highly stereoselective nitrilase from bacteria, actinomycetes, and fugui such as *Rhodococcus rhodochrous* PA-34 and *Aspergillus fumigatus*. The stereoselective synthesis of (*R*)-phenylglycine ((*R*)-2PG) from racemic 2-phenylglycinonitrile (2PGN) was performed using enantioselective nitrilase from *Sphingomonas wittichii* RW1 in combination with *in situ* racemization in aqueous and biphasic aqueous–organic system [9]. Chiral *N*-acyl α-amino acids were also synthesized by asymmetric hydrolysis of racemic *N*-acyl aminonitriles, which were prepared from benzaldehyde by Strecker synthesis and chemical acylation reaction using nitrilase 5086 [10]. These enzymatic methods are useful for enantioselective production of α-amino acids, but an additional chemical step for nitrile synthesis was required and the yields were generally low. In addition, highly stereoselective nitrilases are essential to obtain the enantioselective production.

Nitrile hydratases are attractive biocatalyst for the synthesis of amides from nitriles. For example, nitrile hydratases from *Rhodococcus rhodochrous* J-1, *Rhodococcus* sp. N774 and *Pseudomonas chlororaphis* B23 have been utilized for the industrial synthesis of acrylamide from acrylonitrile [4][11]. A nitrile hydratase from *Rhodococcus* sp. (NOVO SP 361) is also utilized for the synthesis of (*R*)-2-phenylglycine amide ((*R*)-2PGNH$_2$), which is a precursor for the synthesis of β-lactam antibiotics such as Cefalexin from 2PGN [12]. Recently, we developed a method for the asymmetric synthesis of α-amino acids from α-aminonitriles by the dynamic kinetic resolution using nitrile hydratases, amino acid amide racemases, and stereoselective amino acid amide hydrolases [13]. We also developed a new enzymatic method for the synthesis of α-amino acids from amines using a mutant D-amino acid oxidase from porcine kidney (pkDAO Y228L/R283G) and nitrilase AY487533, in which (*R*)-2-methyl-2PG was synthesized with 40% enantiomeric excess (ee) from (*R*)-□α-methylbenzylamine and KCN [14]. This

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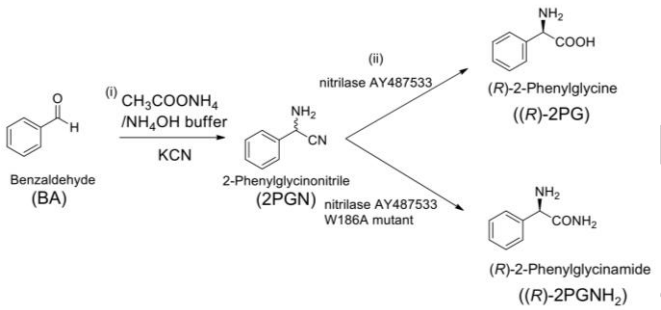
method is unique, because 2-methyl-2PGN is enzymatically synthesized from (*R*)-α-methylbenzylamine and KCN by pkDAO Y228L/R283G, and followed by nitrilase AY487533 reaction in one reaction mixture. However, the method showed rather low enantioselectivity due to the low stereoselectivity of nitrilase AY487533.

Here, we developed a highly enantioselective method for synthesis of α-amino acids from aldehydes and KCN using non-enzymatic Strecker synthesis and nitrilase AY487533 in water. We successfully constructed one-pot reaction system for high enantioselective synthesis of α-amino acids using the kinetic resolution catalyzed by nitrilase AY487533, carefully controlling the low enantioselective nitrilase in the hydrolysis of 2PGN to 2PG. Furthermore, we created a mutant nitrilase AY487533 W186A, which shows a nitrile hydratase-like activity forming amide from α-aminonitrile, and utilized the mutant in the synthesis of (*R*)-2PGNH$_2$ from benzaldehyde and KCN in one-pot reaction. This paper describes the synthesis of (*R*)-2PG and its derivatives utilizing the kinetic characteristics of nitrilase AY487533 from achiral benzaldehyde and KCN. The asymmetric synthesis of (*R*)-2PGNH$_2$ from the same substrates by a mutant nitrilase is also described.



Scheme-1. Chemoenzymatic reaction for synthesis of (*R*)-2PG and (*R*)-2PGNH$_2$ from benzaldehyde using Strecker synthesis and nitrilase AY487533.

Results

Characterization of nitrilase AY487533

The nitrilase AY487533 was identified from metagenome screening by J. A. Chaplin *et al* [15]. They revealed that the enzyme shows nitrile aminohydrolase activity toward 2PGN and 2-methyl-2PGN. We previously report that the optimal temperature and pH of the enzyme activity were 45°C and pH 8.0-9.0, respectively [14]. In this study, we further analyzed the stability and the kinetic characteristics of this enzyme. The enzyme was stable at pH 6.0-10.0 and below 40°C, and at these conditions more than 80% of the original activity after 30 min of incubation (Figure 1). Therefore, the following synthetic reaction of 2PG from 2PGN was performed at pH 8.0.

The kinetic parameters were analyzed under standard assay condition using 0-7.5 mM racemic-2PGN (Figure 2). The K_m value for (*R*)-2PGN was estimated to be 0.097 ± 0.02 mM, which was approximately 12-times lower than that for (*S*)-2PGN (1.19 ± 0.16 mM). The V_{max} value for (*R*)-2PGN in the synthesis of (*R*)-2PG was calculated to be 0.75 ± 0.02 □mol/min/mg protein, which was 8.4-times lower than that for (*S*)-2PGN in the synthesis of (*S*)-2PG (6.28 ± 0.54 □mol/min/mg protein). These results indicate that the enzyme preferably reacts with (*R*)-2PGN, but not with (*S*)-2PGN, in a low concentration of racemic-2PGN. However, when high concentrations of (*R*)- and (*S*)-2PGN are present as substrates, (*S*)-2PGN is rapidly converted into (*S*)-2PG and the resulting (*R*)-2PGN is gradually converted into (*R*)-2PG to some extent, resulting in the synthesis of (*R*)-2PG with 8.7 % ee.

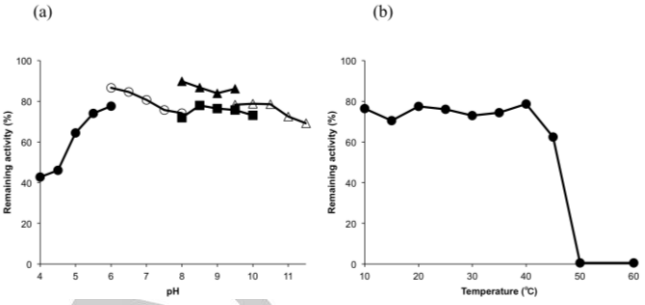


Figure 1. Effects of pH and temperature on stability of nitrilase AY487533. (a) Effect of pH. The enzyme solution was incubated at 20°C for 30 min at pH 5.0 - 11.5 using following buffer solutions. Then, nitrilase activity was assayed under standard assay conditions. The remaining activities were expressed as percentage of the enzyme activity in the absence of heating. ● Citrate buffer (pH 5.0–6.0), ○ KPB (pH 6.0–8.0), ▲ Tris-HCl (pH 8.0–9.5), △ glycine-KOH (pH 9.5–11.5) and ■CH_3COONH_4-NH_4OH (pH 8.0-10.0).
(b) Effect of temperature. The enzyme solution was incubated at pH 8.0 for 30 min at the indicated temperature. Then, nitrilase activity was assayed under standard assay conditions. The remaining activities were expressed as percentage of the enzyme activity in the absence of heating.

Synthesis of 2PG from 2PGN by nitrilase AY487533 reactions

At first, the enantioselective synthesis of 2PG was performed using 10 mM racemic 2PGN and 2U of nitrilase AY487533 at 30°C and pH 8.0. In this reaction, (*S*)-2PG was produced in the early stage and (*R*)-2PG was linearly produced for 30 min. Finally, both (*S*)-2PG and (*R*)-2PG were produced at concentrations up to 5.0 mM and 4.2 mM, respectively, after 30 min of incubation, and ee for (*S*)-2PG was lower than 10% (Figure S4). These results are in agreement with the above kinetic results that nitrilase AY487533 exhibits higher activity toward (*S*)-2PGN at high concentration of the substrate. The results also indicate that racemic 2PGN is not suitable as the starting substrate for the enantioselective production using nitrilase AY487533.

One-pot synthesis of (*R*)-2PG from benzaldehyde and KCN by a combination of Strecker synthesis and nitrilase AY487533 reaction

We next investigated a new method for the enantioselective production of (*R*)-2PG by the combination of Strecker synthesis and nitrilase AY487533 by keeping a low substrate concentration. First, we analyzed the optimal concentration of benzaldehyde and KCN for the enantioselective synthesis of (*R*)-2PG by incubation at 20°C and pH 8.0 for 30 min using 7 U of AY487533, 50-200 mM KCN and 25-150 mM benzaldehyde (Figure S2). In this reaction, (*R*)-2PG was obtained in more than 95% yield and 95% ee when benzaldehyde concentration was kept below 75 mM, and the yields gradually decreased upon increasing the benzaldehyde concentration. The KCN concentration also affected both the yield and ee for (*R*)-2PG

production, and 150 mM KCN being the optimal concentration. On the basis of these results, 50 mM benzaldehyde and 150 mM KCN were incubated at 20ºC and pH 8.0 in the presence of 7 U of AY487533. In this reaction condition, 48 mM (*R*)-2PG was produced with 98 % ee after 30 min of incubation (Figure 3 and Table 1). In addition, 2PGN was not detected during the reaction (data not shown). These results indicated that racemic 2PGN synthesized by Strecker synthesis in the reaction mixture was immediately converted to (*R*)-2PG by nitrilase AY487533, and high concentration of (*R*)-2PG was produced with high ee. As mentioned above, when high concentration of racemic 2PGN was used as a starting substrate, the yield and ee of the product (*R*)-2PG was very low due to the low V_{max} value and low stereoselectivity of nitrilase AY487533. On the other hand, when benzaldehyde and KCN were used as starting substrates to perform Strecker synthesis and nitrilase reaction in the same reaction mixture, almost all of benzaldehyde was converted into (*R*)-2PG *via* 2PGN.

These results strongly indicate that the racemic 2PGN was synthesized at pH 8.0 in a slower process yielding low concentration of the product by chemical Strecker synthesis. The resulting (*R*)-2PGN was preferentially converted into (*R*)-2PG by nitrilase AY478533, because of the high affinity of the enzyme toward (*R*)-2PGN. However, (*S*)-2PGN is not a good substrate for the nitrilase because of the high K_m value for (*S*)-2PGN.

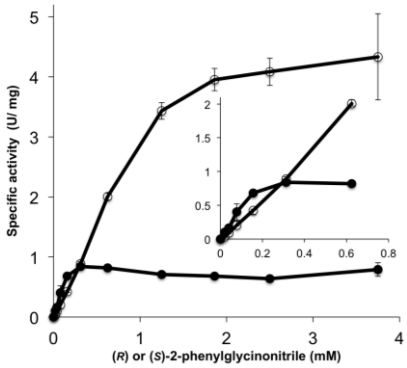


Figure 2. Saturation curve of nitrilase AY487533 for (*R*)-2PG and (*S*)-2PG production.
The nitrilase reaction velocities were measured in the standard assay conditions at 30°C for 5 min with 0.04-7.5 mM racemic-2PGN and the nitrilase activities for production of (*R*)-2PG and (*S*)-2PG were determined by HPLC analysis. ●: (*R*)-2PG, ○ : (*S*)-2PG

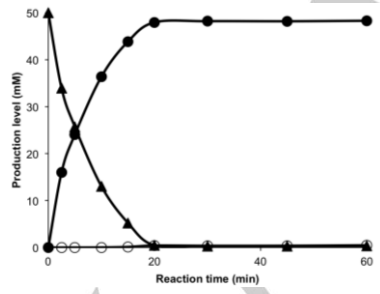


Figure 3. Time course of the asymmetric synthesis of (*R*)-2PG from benzaldehyde and KCN.
The substrate solution containing 50 mM benzaldehyde and 150 mM KCN was incubated with 7 U of crude enzyme solution of nitrilase AY487533 in CH_3COONH_4-NH_4OH buffer, pH 8.0, at 20°C. ●: (*R*)-2PG, ○ : (*S*)-2PG , ▲: Benzaldehyde

One-pot synthesis of (*R*)-2PG derivatives from benzaldehyde derivatives and KCN

As mentioned above, we demonstrated that (*R*)-2PG was synthesized from benzaldehyde and KCN with high yield and ee by the combination of Strecker synthesis and nitrilase AY487533 reaction. The method was applied to the synthesis of (*R*)-2PG derivatives. When 50 mM 4-hydroxybenzaldehyde was incubated with 150 mM KCN and 7 U of nitrilase AY487533 under the same reaction conditions described above, (*R*)-4-hydroxy-2PG was synthesized with 97.8% of yield with 98.4% ee. (*R*)-4-Fluoro-2PG was also produced from 50 mM 4-fluorobenzaldehyde with 93.6% of yield with 98.7% ee using the same reaction conditions (Table 1).

Construction of nitrile hydratase from nitrilase AY487533 by mutation at W186

Sosedov and Stolz reported that nitrilase from *Pseudomonas fluorescens* EBC191 was mutated to an enzyme exhibiting nitrile hydratase activity by site-directed mutagenesis of Trp residue at position 188, and the mutant nitrilase was applied to perform the synthesis of mandeloamide from mandelonitrile [16]. They also predicted the five amino acid residues (E48, K130, E137, C164, and W188) are important for residues to show the nitrilase activity. Based on the example, we analyzed the similarity of the amino acid sequences between nitrilase AY487533 and the one from *P. fluorescens* EBC191. The amino acid sequence of nitrilase AY487533 showed 57% identity with that of nitrilase from *P. fluorescens* EBC191. Interestingly, the five important amino acids were conserved at following positions in nitrilase AY487533- E46, K128, E135, C162 and W186 (Figure S5). Therefore, we performed saturation mutagenesis of nitrilase AY487533 at W186 and discovered the results that mutant W186A showed a high activity for the production of $2PGNH_2$ from 2PGN using high-performance liquid chromatography (HPLC) analysis (Figure S6). We next utilized the AY487533 W186A variant for the synthesis of 2-$PGNH_2$ from benzaldehyde and KCN by the combination with Strecker synthesis in the same reaction mixture. Using this variant (7U of cell-free extract for 1mL of reaction), 39.7 mM (*R*)-$2PGNH_2$ was synthesized from 50 mM benzaldehyde and 150 mM KCN by incubation with 500 mM CH_3COONH_4-NH_4OH buffer, at pH 8.0, and 20ºC for 60 min (79.5% yield and 86.5% ee) (Figure 4 and Table 1). In this reaction, the production of 2-PG was less than 7.2 mM. This result indicates that our new method is applicable for preferential production of (*R*)-$2PGNH_2$ from benzaldehyde and KCN by using AY487533 W186A variant.

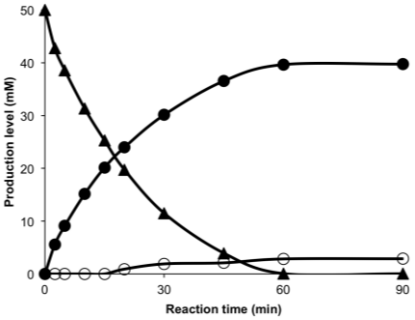


Figure 4. Time course of the asymmetric synthesis of (*R*)-$2PGNH_2$ from benzaldehyde and KCN.

The substrate solution containing 50 mM benzaldehyde and 150 mM KCN was incubated with 7 U of crude enzyme solution of nitrilase AY487533 W186A in CH_3COONH_4-NH_4OH buffer (pH 8.0), at 20°C. The substitute solution containing 150 mM KCN and 50 mM benzaldehyde in CH_3COONH_4/NH_4OH buffer (pH 8.0) was incubated with at 20°C. ●: (*R*)-2PGNH_2, ○: (*S*)-2PGNH_2, ▲: Benzaldehyde

Table 1. The derivative of (*R*)-2PG from corresponding benzaldehyde using Strecker synthesis and nitrilase AY487533.

Product	Yield (%)	ee (%)
(*R*)-2-phenylglycine	96.5	98.5
(*R*)-4-OH-2PG	97.8	98.4
(*R*)-4-F-2PG	93.6	98.7
(*R*)-2-phenylglycinamide	79.5	86.5

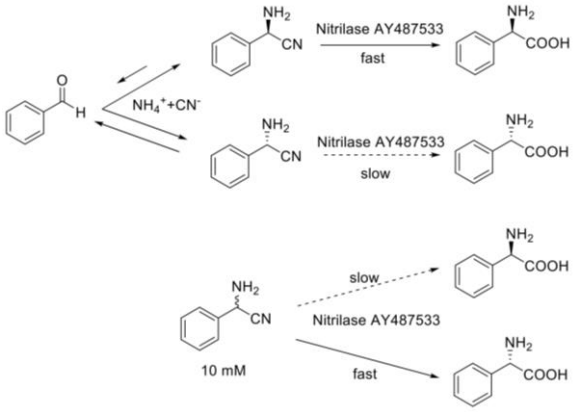
ee (%)= {((*R*)-product (mM)-(*S*)-product (mM))/ ((*R*)-product (mM) +(*S*)-product (mM)) }×100

Discussion

Chiral α-amino acids are important intermediates for the production of pharmaceuticals and fine chemicals. Therefore, the methods for enantioselective synthesis of chiral amino acids and their derivatives have been widely investigated using chemical, chemoenzymatic and enzymatic approaches. Among these, Strecker synthesis is the most important chemical method to prepare racemic α-aminonitriles from aldehydes or ketones and KCN. The corresponding amino acids can be easily obtained by hydrolysis. Thus, Strecker synthesis is useful for the achiral amino acid synthesis followed by several enzymatic kinetic resolution procedures. However, industrially effective method of chiral amino acid synthesis using combined with catalyst and Sterecker reaction in one pot system is unavailable, because highly enantioselective nitrilases or chemical catalysts are required to obtain chiral amino acids. The separation synthesis methods of Strecker synthesis and stereoselective hydration also have disadvantages such as requirement of more than two different reaction vessels to obtain chiral products, and low yield. In addition, the screening or construction of such high enantioselective enzymes is not easy. For example, we previously developed nitrile hydratase with low enantioselectivity to construct a synthetic method for amino acids in combination with an amino acid amide racemase and stereoselective amidases, because stereoselective nitrile hydratases are hard to obtain [4]. A highly stereoselective nitrilase from *Alcaligenes faecalis* ATCC 8750 was discovered and utilized for enantioselective hydrolysis of racemic mandelonitrile to (*R*)-(-)-mandelic acid, a versatile chiral building block [17]. (*S*)-Enantioselective hydrolysis of racemic α-aminonitile was made possible with a nitrilase from *Rhodococcus rhodochrous* PA-34 [18]. L-form amino acids were produced except for alanine. Enzymatic cascade synthesis of (*S*)-2-hydroxy carboxylic amide and its acid from aldehydes was performed by hydroxynitrile lyase and nitrile-converting enzymes. [19] The cascade reaction needed two enzymes and the production level was low (less than 10 mM).

To solve the problems with previous methods, we investigated a new attractive chemo-enzymatic method for highly enantioselective production of (*R*)-amino acids using a low enantioselective nitrilase. In this study, the enantioselective production of (*R*)-2PG, which is useful as the side chain of antibiotics like amoxicillin [20], was used as an example to propose the new method. When 10 mM racemic 2PGN was directly incubated with nitrilase AY487533 according to the conventional methods, the production velocity of (*S*)-2PG was faster than that of (*R*)-2PG, and 5.0 mM (*S*)-2PG and 4.2 mM (*R*)-2PG were finally produced (8.7% ee) (Figure S4), showing a low enantioselectivity of the enzyme. However, when the same enzyme was used in our one-pot method, 48 mM (*R*)-2PG was enantioselectively produced from 50 mM benzaldehyde with 98 % ee upon 30 min of incubation (Figure 3 and Table 1). The kinetic analysis of this enzyme showed that affinity of the enzyme to (*R*)-2PGN is much higher than to (*S*)-2PGN, whereas the V_{max} value for (*R*)-2PGN to produce (*R*)-2PG is much lower than that for (*S*)-2PGN. Therefore, we understand that the concentration of racemic 2PGN strongly affected the enantioselectivity of the products by nitrilase AY487533 reaction and a low concentration of (*R*)-2PGN is needed for highly enantioselective production of (*R*)-2PG. Thus, it is understood that (*R*)-2PGN in racemic 2PGN, which is produced by Strecker synthesis in the same reaction mixture with nitrilase AY487533, is preferentially converted to (*R*)-2PG by nitrilase AY487533 under the low concentration of racemic 2PGN which is produced by the very slow Strecker synthesis reaction. These results also indicate that (*S*)-2PGN synthesized by Strecker synthesis is dynamically converted back to racemic 2PGN by retro-Strecker synthesis, while (*R*)-2PGN is consumed to form (*R*)-2PG, which accumulates with high ee, by the nitrilase reaction. These speculations are also supported by the experimental results that (*S*)-2PGN was not detected during the synthesis reaction. The data also suggests that concentration of (*S*)-2PG is keeping lower by the relatively slow Strecker synthesis is a key point in the highly enantioselective production of (*R*)-2PG by our method (Scheme-2). Therefore, we concluded that highly enantioselective production of (*R*)-2PG is achieved by higher affinity of nitrilase AY487533 for the (*R*)-enantiomer. Applicability of our proposed method was confirmed by the synthesis of (*R*)-2PG derivatives using nitrilase AY487533 and of (*R*)-2PGNH_2 using nitrilase AY487533 W186A variant, because these products are also enantioselectively synthesized with high yield.

Thus, our proposed method has following advantages: 1) The sequential reactions of Strecker synthesis and nitrilase reaction are performed in the same reaction mixture. 2) Both Strecker synthesis and nitrilase reaction are performed under mild conditions, which are similar to the enzyme reaction. 3) Aldehydes or ketones and KCN can be used as the starting substrates without synthesis of nitriles. 4) Low enantioselective enzymes can be utilized in our method. 5) Chiral products are synthesized with high ee and yield, even though low enantioselective enzymes are used. Therefore, our methods are attractive and open a new door for the enantioselective synthesis of chiral products. We expect that our methods will contribute to the construction of simple methods for enantioselective synthesis by using other enzymes with different substrate specificities.



Scheme-2. Mechanism of the chemoenzymatic reaction using nitrilase AY487533.

Conclusions

We developed a new chemoenzymatic method for the enantioselective synthesis of (*R*)-2PG from benzaldehyde and KCN by sequentially performing the Strecker reaction and nitrilase reaction in the same reaction mixture. The method is based on the high affinity of enzyme to one enantiomer, and can be utilized for one-pot synthesis of target chiral compounds. The same principle of the method also was utilized for the enantioselective synthesis of (*R*)-2PGNH$_2$ from benzaldehyde and KCN with the mutant nitrilase W186A mutant. Thus, we demonstrated how low enantioselective enzymes could also be used for enantioselective synthesis of target products. Our methods can be widely utilized to reduce the number of steps in enantioselective synthesis.

Experimental Section

Materials

Benzaldehyde, (*R*)- and (*S*)-2PG methylester and racemic 2PGN hydrochloride were purchased from Sigma-Aldrich Japan (Tokyo, Japan). (*R*)- and (*S*)-2PG methylester were used for synthesis of (*R*)- and (*S*)-2PGNH$_2$ in ammonia solution, respectively. (*R*)- and (*S*)-2PG, racemic-4-fluoro-2PG, (*R*)- and (*S*)-4-hydroxy-2PG and racemic-4-chloro-2PG were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). (*R*)-4-fluoro-2PG and organic solvent for HPLC were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of commercially available grade. The ligation mixture, polymerases, restriction enzymes and site-directed mutagenesis kit were obtained from Takara Bio (Otsu, Japan) for gene cloning and mutagenesis of nitrilase AY487533.

Assay for the enzyme activity of nitrilase and nitrile hydratase

Nitrilase activity was assayed by measuring the amount of (*R*)- and (*S*)-2PG produced from racemic 2PGN by following reaction. The substrate solution (0.5 ml) containing 20 mM 2PGN in 100 mM potassium phosphate buffer (KPB), pH 8.0, was pre-incubated at 30°C for 5 min, and the reaction was initiated by adding the enzyme solution (0.02 ml). The reaction was performed at 30°C for 5 min, and stopped by adding 0.1 mL of 2 M $HClO_4$. The resulting precipitates were separated by centrifugation. Then, the amount of 2PG in the supernatant was analyzed by HPLC. One unit of nitrilase activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of (*R*) and (*S*)-2PG from 2PGN per min.

Nitrile hydratase activity could not be assayed using same condition of HPLC because of the overlapping products and substrate peaks were not separate in HPLC.

Analysis of reaction products by HPLC

The reaction products were analyzed by HPLC (Waters, Tokyo, Japan) with a Crownpack CR-I (+) column (Daicel Co., Osaka, Japan) at 25°C using a solution containing 60 mM $HClO_4$ and acetonitrile (80:20) at a flow rate of 0.4 ml/min. The product amounts were measured at 200 nm using a UV detector.

Purification of nitrilase AY487533

E. coli BL21 (DE3) cells harboring pET15b-*nitrilase AY487533* were cultivated at 37 °C for 12 h in 5 mL of LB medium containing 100 μg / mL ampicillin. The grown cells (5 mL) were then transferred into 500 mL of LB medium containing 100 μg / mL ampicillin and cultivated at 37 °C for 3 h. Then, 0.5 mM IPTG was added, and further incubated at 30 °C for 6 h. The cells (25 g wet weight) were harvested by centrifugation (5000 × g, 15 min), and washed with 20 mM KPB, pH 8.0. Then, the cells were disrupted by sonication, and the cell debris was removed by centrifugation (20,000 × g, 30 min, 4 °C). Then, solid ammonium sulfate was added to the supernatant up to 20% saturation, and the resulting precipitate upon centrifugation was discarded. Solid ammonium sulfate was further added into the supernatant solution up to 35% saturation. The precipitate formed was collected, and dissolved with 10 mM KPB (pH 8.0) containing 0.1% 2-mercaptoethanol (2ME) and then dialyzed against the same buffer. The dialyzed enzyme solution was applied to a DEAE-Toyopearl column (10 cm × 2.8 cm diameter) equilibrated with 10 mM KPB, pH 8.0, containing 0.1% 2-ME, and the adsorbed enzyme was eluted by a linear gradient of NaCl (0–0.5 M, 500 mL each). The active fractions were collected and ammonium sulfate was added to the active fractions up to 20% saturation. The precipitant was collected after centrifugation (20 000 × g, 30 min, 4 °C) and dissolved with 100 mM KPB (pH 8.0) containing 0.1% 2-ME. As the enzyme showed a single protein band on SDS-PAGE, it was used for the studies of enzyme stability and kinetic analysis (Figure S1).

Screening for nitrilase AY487533 enzyme mutated at W186 residue

The saturation mutagenesis of the nitrilase was performed at W186 residue using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with primers containing the NNK codon and the pET15-*Nitrilase AY487533* as a template, according to the manufacturer's instructions, as follows. The reaction mixture consisted of 1 μL of 10 × reaction buffer, 0.2 μL of dNTP mix, 7.7 μL of distilled water, 2.5 U of *PfuTurbo* DNA polymerase, 0.15 μL (10 ng/μL) of the sense and antisense primers, and 10 ng of pET15b-nitrilase AY487533 vector as template DNA. The following steps were performed for 18 cycles: a denaturing step at 95ºC for 20 sec (first cycle at 95ºC for 2 min), an annealing step at 60ºC for 10 sec and an elongation step at 68ºC for 4 min. The product was treated with 10 U of *Dpn*I at 37ºC for 1 h, and then used for transformation of *E. coli* BL21 (DE3) using the heat-shock method. The mutants were cultivated at 37 °C for 12 h in 5 ml of LB medium containing 100 μg / mL ampicillin. The grown cells (5 mL) were then transferred into 5 ml of LB medium containing 100 μg / mL ampicillin and cultivated at 37 °C for 3 h. Then, 0.5 mM IPTG was added to the culture, followed by the incubation at 30 °C for 6 h. The cells were harvested by centrifugation (5000 × g, 15 min), and washed with 20 mM KPB, pH 8.0. Then, the cells were disrupted by sonication, and the cell debris was removed by centrifugation (20,000 × g, 20 min, 4 °C). Using the cell-free extract, 2PGNH$_2$ production from 2PGN was carried out by incubation at 30°C for 10 min with 20 mM 2PGN in 100 mM KPB (pH 8.0). The active enzyme leading to the 2PGNH$_2$ production was selected to assay the 2PGNH$_2$ using Thin-Layer chromatography (TLC) and HPLC.

Preparation of crude enzyme solution of mutant nitrilase AY487533 W186A

E. coli BL21 (DE3) cells harboring pET15b-nitrilase AY487533 *W186A* were cultivated under the same conditions as cells harboring pET15b-nitrilase AY487533. The cells cultivated at 37 °C for 6 h in 500 mL of LB medium were harvested by centrifugation and washed with 20 mM KPB (pH 8.0). Then, the cells were disrupted by sonication, and the supernatant solution was used for the synthesis of amides.

Effects of pH and temperature on nitrilase AY487533 activity

The nitrilase activity was assayed under standard assay conditions, except that the reaction pH was varied between pH 5.0 and 11.5 using Citrate buffer (pH 5.0–6.0), KPB (pH 6.0–8.0), Tris-HCl (pH 8.0–9.5), CH_3COONH_4-NH_4OH (pH 8.0-10.5), and glycine-KOH (pH 9.5–11.5).

Effect of temperature on nitrilase activity was assayed under standard assay conditions, except that the temperature was varied between 10 and 80 °C.

Effects of pH and temperature on nitrilase AY487533 stability

The effect of pH on enzyme stability was analyzed between pH 5.0 and 11.5 by incubation at 30°C for 30 min without the substrate using the same buffers as described above. The effect of temperature on enzyme stability was assayed by incubation at 10-80 °C with 100 mM KPB (pH 8.0), for 30 min without the substrate. The enzyme activity was assayed under standard assay conditions.

One-pot synthesis of (*R*)-2PG from benzaldehyde and KCN by a combination of Strecker synthesis and nitrilase AY487533

The synthesis of (*R*)-2PG from benzaldehyde and KCN by one-pot chemo-enzymatic reaction using Strecker synthesis and nitrilase AY487533 was performed as follows. The reaction mixture (0.5 mL) containing 50 mM benzaldehyde, 150 mM KCN and crude enzyme solution of nitrilase AY487533 was incubated at 20°C for 60 min with 500 mM CH_3COONH_4-NH_4OH buffer, pH 8.0. The reaction was stopped by addition of 0.1 mL of 2 M $HClO_4$, and precipitate formed upon centrifugation was discarded. The reaction products in the supernatant were analyzed by HPLC with Crownpack CR-I (+). The same reaction conditions were also used for synthesis of 4-hydroxy-2PG from 4-hydroxy-benzaldehyde and 4-fluoro-2PG from 4-fluoro-benzaldehyde.

One-pot synthesis of (*R*)-2PGNH_2 from benzaldehyde and KCN

The synthesis of (*R*)-2PGNH_2 from benzaldehyde and KCN by one-pot chemoenzymatic reaction was performed under the same reaction conditions as the synthesis of (*R*)-2PG by using crude enzyme solution of nitrilase AY487533 W186A. The reaction products were analyzed using HPLC as described above.

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Keywords: nitrile, amino acid, Strecker synthesis, nitrilase

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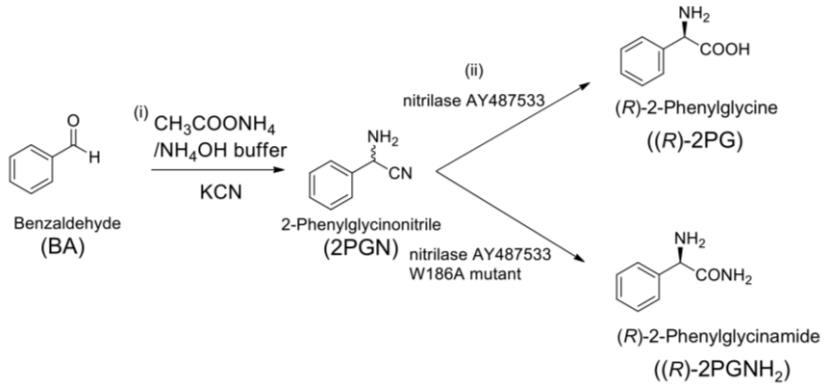
FULL PAPER

Chemoenzymatic reaction for synthesis of (*R*)-2PG and (*R*)-2PGNH2 from benzaldehyde using Strecker synthesis and nitrilase AY487533.



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A novel chemoenzymatic method for enantioselective synthesis of (*R*)-