Enzymes Responsible for the Conversion of N^{α}-[(Benzyloxy)carbonyl]-D-lysine to N^{α}-[(Benzyloxy)carbonyl]-D-aminoadipic Acid by *Rhodococcus* sp. AIU Z-35-1

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The enzymes responsible for the conversion of N^{α}-[(benzyloxy)carbonyl]-D-lysine (N^{α}-Z-D-lysine) to N^{α}-Z-D-aminoadipic acid (N^{α}-Z-D-AAA) by *Rhodococcus* sp. AIU Z-35-1 were identified. N^{α}-Z-D-Lysine was first converted to N^{α}-Z-D-aminoadipic δ-semialdehyde (N^{α}-Z-D-AASA) by D-specific amino acid deaminase, whereas N^{α}-Z-L-lysine was converted to N^{α}-Z-L-AASA by L-specific amino acid oxidase. The resulting N^{α}-Z-D-AASA was then converted to N^{α}-Z-D-AAA by the same aldehyde dehydrogenase that is responsible for N^{α}-Z-L-AASA oxidation. The product amount of the D-specific amino acid deaminase reached the maximum at one day of cultivation in the L-lysine medium. The aldehyde dehydrogenase reached the maximum at three days of cultivation.

Introduction. – L-α-Aminoadipic δ-semialdehyde (L-α-AASA) and L-α-aminoadipic acid (L-α-AAA) are precursors of biosynthesis of β-lactam antibiotics, and both compounds and their derivatives provide interesting raw materials for the chemical synthesis of pharmaceuticals or physiologically active peptides. Some biochemical methods for the production of L-α-AASA, L-α-AAA, and their related compounds have been developed using L-lysine-6-aminotransferase or L-lysine-6-dehydrogenase [1][2]. However, these methods had some drawbacks such as the requirement of a second substrate or cofactor regeneration system, and the yields were low. We, therefore, isolated a new bacterial strain, *Rhodococcus* sp. AIU Z-35-1, as a high producer of N^{α}-Z-L-AAA from N^{α}-Z-L-lysine, to overcome the above drawbacks, and developed an efficient method for production of N^{α}-Z-L-AAA by the cell reaction [3]. This strain was also useful for the production of N^{α}-Z-L-AASA, N^{α}-Z-D-AASA, and N^{α}-Z-D-AAA, since N^{α}-Z-L-lysine was converted to N^{α}-Z-L-AAA *via* N^{α}-Z-L-AASA and N^{α}-Z-D-lysine was converted to N^{α}-Z-D-AAA *via* N^{α}-Z-D-AASA [3][4]. Recently, we identified the enzymes catalyzing the conversion of N^{α}-Z-L-lysine to N^{α}-Z-L-AAA [5][6]. However, the enzymes responsible for the conversion of N^{α}-Z-D-lysine to N^{α}-Z-D-AAA have not been identified. Therefore, the present article describes the identification of the enzymes catalyzing the conversion of N^{α}-Z-D-lysine to N^{α}-Z-D-AAA. The optimal cultivation time for the production of these enzymes was also determined.

Results and Discussion. – *Identification of the Enzyme Catalyzing the Conversion of* N^{α}*-Z-D-Lysine to* N^{α}*-Z-D-AASA.* Cells from *Rhodococcus* sp. AIU Z-35-1, harvested from the L-lysine medium after 1 d of cultivation, were incubated with N^{α}-Z-D-lysine,

and the reaction products were analyzed. In this reaction, formation of N^{α}-Z-D-AASA and NH_3, but not of H_2O_2, was observed (data not shown). When the crude enzyme solution was incubated with N^{α}-Z-D-lysine, formation of N^{α}-Z-D-AASA and NH_3 was also detected, but that of H_2O_2 and NAD(P)H was not (data not shown). In addition, the produced amount of N^{α}-Z-D-AASA increased in parallel with that of NH_3 (*Fig. 1*). Then, the stereospecificity of the enzyme was analyzed using the partially purified enzyme. The formation of NH_3 was detected from N^{α}-Z-D-lysine, but not from N^{α}-Z-L-lysine. These results indicate that the conversion of N^{α}-Z-D-lysine to N^{α}-Z-D-AASA was catalyzed by a D-specific amino acid deaminase in *Rhodococcus* sp. AIU Z-35-1, according to *Scheme 1*.

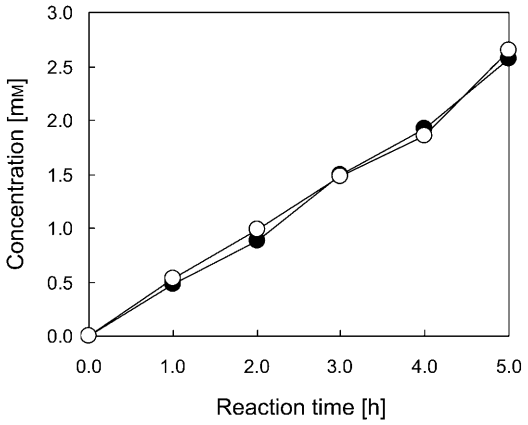
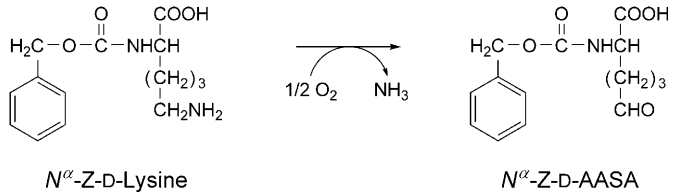


Fig. 1. *Formation of NH_3 and* N^{α}-*Z*-D-*AASA from* N^{α}-*Z*-D-*lysine.* N^{α}-Z-D-Lysine (30 μmol) was incubated with 0.009 units of D-amino acid deaminase at 30° and pH 7.0. Open circles, N^{α}-Z-D-AASA; closed circles, NH_3.

Scheme 1. *Conversion of* N^{α}-*Z*-D-*Lysine to* N^{α}-*Z*-D-*AASA by* Rhodococcus *sp. AIU Z-35-1*



N^{α}-Z-D-Lysine N^{α}-Z-D-AASA

We have already reported [5] that when N^{α}-Z-L-lysine was used as substrate, its conversion to N^{α}-Z-L-AASA was catalyzed by an oxidase purified from *Rhodococcus* sp. AIU Z-35-1 and that the amount of H_2O_2 produced increased in parallel with that of NH_3 and N^{α}-Z-L-AASA. We further revealed that this enzyme also exhibited oxidase activity on N^{α}-acetyl-L-lysine, N^{ε}-acetyl-L-lysine, and L-amino acids, except for glycine, L-proline, and L-cysteine, but not on N^{α}-Z-D-lysine, N^{α}-acetyl-D-lysine, N^{ε}-acetyl-D-lysine, and D-amino acids [5]. Thus, N^{α}-Z-L-lysine was converted to N^{α}-Z-L-AASA by the L-specific amino acid oxidase with broad substrate specificity in *Rhodococcus* sp. AIU Z-35-1.

In our studies on the enzymes responsible of the conversion of N^{α}-Z-D-lysine to N^{α}-Z-D-AASA and N^{α}-Z-L-lysine to N^{α}-Z-L-AASA, it was concluded that N^{α}-Z-D-lysine was converted to N^{α}-Z-D-AASA by the D-specific amino acid deaminase, whereas N^{α}-Z-L-lysine was converted to N^{α}-Z-L-AASA by the L-specific amino acid oxidase in *Rhodococcus* sp. AIU Z-35-1. We have already revealed in an earlier report [7] that N^{α}-Z-L-lysine and N^{α}-Z-D-lysine were converted to N^{α}-Z-L-AASA and N^{α}-Z-D-AASA, respectively, by amine oxidase from *Aspergillus niger* AKU 3302. Thus, the enzyme catalyzing the conversion of N^{α}-Z-D-lysine to N^{α}-Z-D-AASA in the *Rhodococcus* strain was also different from that of *A. niger*.

Identification of the Enzyme Catalyzing the Conversion of N$^{\alpha}$-*Z*-D-*AASA to* N$^{\alpha}$-*Z*-D-*AAA*. When the crude enzyme solution, which was prepared from the cells incubated in the L-lysine medium, was incubated with N^{α}-Z-D-AASA, no formation of H_2O_2 and N^{α}-Z-D-AAA was detected. However, formation of N^{α}-Z-D-AAA was observed when NAD^+ was added to the solution. Moreover, the produced amount of NADH increased in parallel with that of N^{α}-Z-D-AAA (*Fig. 2*). Thus, it was concluded that N^{α}-Z-D-AASA was converted to N^{α}-Z-D-AAA by aldehyde dehydrogenase, according to *Scheme 2*. Then, the substrate specificity of this aldehyde dehydrogenase was analyzed with the purified enzyme. This enzyme also exhibited dehydrogenase activity towards N^{α}-Z-L-AASA. These results indicated that the enzyme catalyzing the conversion of

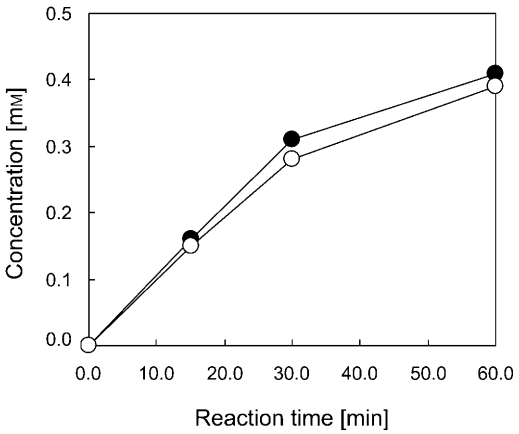
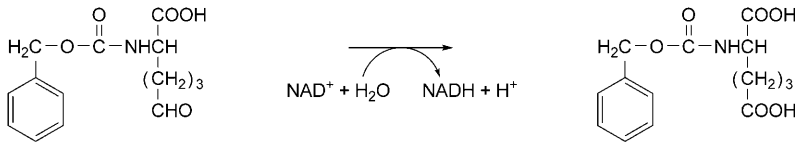


Fig. 2. *Formation of NADH and* N$^{\alpha}$-*Z*-D-*AAA from* N$^{\alpha}$-*Z*-D-*AASA*. N^{α}-Z-D-AASA (30 μmol) was incubated with 0.02 units of aldehyde dehydrogenase at 30° and pH 7.0. Open circles, N^{α}-Z-D-AAA; closed circles, NADH.

Scheme 2. *Conversion of* N$^{\alpha}$-*Z*-D-*AASA to* N$^{\alpha}$-*Z*-D-*AAA by* Rhodococcus *sp. AIU Z-35-1*



N^{α}-Z-D-AASA N^{α}-Z-D-AAA

N^{α}-Z-D-AASA to N^{α}-Z-D-AAA did not show stereospecificity, and both conversions N^{α}-Z-D-AASA to N^{α}-Z-D-AAA and N^{α}-Z-L-AASA to N^{α}-Z-DLAAA were catalyzed by the same aldehyde dehydrogenase in *Rhodococcus* sp. AIU Z-35-1.

Production of Enzyme. Rhodococcus sp. AIU Z-35-1 was incubated in the L-lysine medium at 30° for four days, and the N^{α}-Z-D-lysine deaminase activity and the N^{α}-Z-D-AASA dehydrogenase activity were assayed each day using cell-free extract. The N^{α}-Z-D-lysine deaminase activity reached the maximum at 1 d of cultivation and was reduced by a longer cultivation time. In contrast, the N^{α}-Z-D-AASA dehydrogenase activity reached the maximum at three days of cultivation (*Fig. 3*). These results were in good agreement with our previous results, which indicated that a selective production of N^{α}-Z-D-AASA was achieved using the cells harvested after one day of cultivation in the L-lysine medium and that N^{α}-Z-D-AAA was efficiently produced using the cells harvested after two days of cultivation.

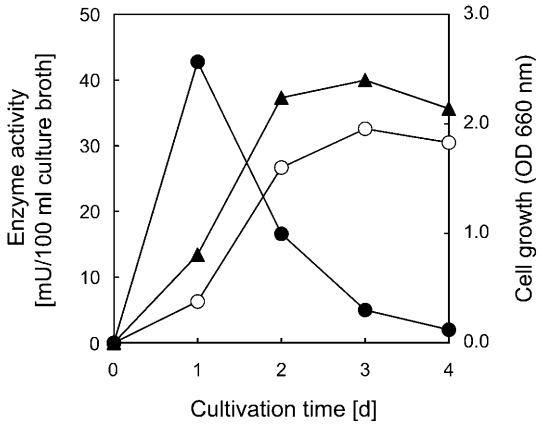


Fig. 3. *Production of* D-*amino acid deaminase and aldehyde dehydrogenase. Rhodococcus* sp. AIU Z-35-1 was incubated in the L-lysine medium at 30° for 4 d, and the D-amino acid deaminase and the aldehyde dehydrogenase activities were assayed each day using cell-free extract. Closed circles, D-amino acid deaminase; open circles, aldehyde dehydrogenase; triangles, cell growth (OD_{660} nm).

Conclusions. – To identify the enzymes catalyzing the conversion of N^{α}-Z-D-lysine to N^{α}-Z-D-AAA in *Rhodococcus* sp. AIU Z-35-1, a crude enzyme solution from the cells cultivated in the L-lysine medium were prepared, and the enzyme activities towards N^{α}-Z-D-lysine and N^{α}-Z-D-AASA were assayed. When N^{α}-Z-D-lysine was used as substrate, formation of NH_3, but not of H_2O_2 and NAD(P)H, was observed. In addition, the product amounts of NH_3 and N^{α}-Z-D-AASA were stoichiometrically increased by the reaction with partially purified enzyme. This enzyme did not catalyze the deamination of N^{α}-Z-L-lysine. It was, therefore, concluded that the conversion of N^{α}-Z-D-lysine to N^{α}-Z-D-AASA was catalyzed by D-specific amino acid deaminase in *Rhodococcus* sp. AIU Z-35-1.

When N^{α}-Z-D-AASA was incubated with the crude enzyme solution, formation of NADH and N^{α}-Z-D-AAA, but not of H_2O_2, was observed. This enzyme also exhibited dehydrogenase activity towards N^{α}-Z-L-AASA. It was therefore concluded that N^{α}-Z-

D-AASA was converted to N^{α}-Z-D-AAA by non-stereospecific aldehyde dehydrogenase.

In our previous studies of the enzymes responsible for the production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA from N^{α}-Z-L-lysine by the cells of *Rhodococcus*, we have revealed that N^{α}-Z-L-AASA was produced from N^{α}-Z-L-lysine by L-specific amino acid oxidase, and the resulting N^{α}-Z-L-AASA was converted to N^{α}-Z-L-AAA by aldehyde dehydrogenase. It was therefore concluded that N^{α}-Z-L-AAA and N^{α}-Z-D-AAA were produced from N^{α}-Z-L-lysine and N^{α}-Z-D-lysine by the combination of L-amino acid oxidase and non-stereospecific aldehyde dehydrogenase, and by D-amino acid deaminase and non-stereospecific aldehyde dehydrogenase, respectively, in *Rhodococcus* sp. AIU Z-35-1.

Experimental Part

Chemicals. N^{α}-[(Benzyloxy)carbonyl]-D-lysine (N^{α}-Z-D-lysine) and N^{α}-Z-L-lysine were purchased from *Watanabe Chemical Industries* (Hiroshima, Japan), and β-NAD^+ and $NADP^+$ were obtained from *Oriental Yeast* (Osaka, Japan). N^{α}-Z-D-aminoadipic δ-semialdehyde (N^{α}-Z-D-AASA) and N^{α}-Z-L-AASA were prepared according to the method described in [4]. Peroxidase was a gift from *Amano Enzyme* (Nagoya, Japan). All other chemicals used were of analytical grade and commercially available.

Cultivation of Microorganisms. Rhodoccocus sp. AIU Z-35-1 was incubated in an L-lysine medium at 30° for 1 or 2 d with shaking at 120 strokes per min, according to the method of *Isobe et al.* [3]. The cells were harvested by centrifugation at 20,000*g* for 10 min, washed with 0.1M potassium phosphate buffer pH 7.0, and stored at −20° until use.

Purification of Enzymes. The cells cultivated in the L-lysine medium were suspended in 1.0 ml of 10 mM buffer (pH 7.0) and disrupted with glass beads using a multi-bead shocker (*Yasui Kikai*, Osaka) at 2500 rpm for 8 min (4 × 2 min). The supernatant was obtained by centrifugation at 20,000*g* for 10 min and used as crude enzyme soln. The crude enzyme soln. was applied to column chromatography (CC; *DEAD-Gigacap Q* (*Tosoh*, Tokyo, Japan) and hydroxyapatite). The eluate from the hydroxyapatite column was used as a partially purified D-amino acid deaminase.

Aldehyde dehydrogenase was purified from the crude enzyme soln. by CC (*DEAE-Toyopearl*, *Phenyl-Toyopearl*, and *Blue-Sepharose*), according to the method of *Isobe et al.* [6].

Analysis of Reaction Products. The reaction products were identified by HPLC using a *TSK-Gel DEAE-5PW* column (*Tosoh*, Tokyo, Japan), according to the method of *Isobe et al.* [8]. The product was collected by monitoring the absorbance at 210 nm, and the molecular mass was then analyzed by a *Finnigan Mass Spectrometer LCQ Deca* (*Thermo Electron*, Yokohama, Japan). The aldehyde group of the product was confirmed using 3-methyl-2-benzothiazolinone hydrazone, according to the method of *Paz et al.* [9]. The amounts of reaction products were calculated from the peak area of the HPLC chromatograms obtained with the *TSK-Gel DEAE-5PW* column under the same conditions as used for the identification of the reaction products.

Assay of Enzyme Activity. The oxidase activity was assayed by measuring the rate of H_2O_2 formation as follows. The standard mixture contained 40 μmol of substrate, 0.6 μmol of 4-aminoantipyrine, 1.94 μmol of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt dihydrate, 6.7 units of peroxidase, 0.1 mmol of potassium phosphate (pH 7.0), and an appropriate amount of enzyme in a final volume of 1.0 ml. The color development by H_2O_2 formation was spectrophotometrically followed at 30° by measuring the absorbance at 555 nm [5].

The dehydrogenase activity was spectrophotometrically assayed by measuring the rate of NADH formation as follows. The standard mixture contained 20 μmol of substrate, 0.6 μmol of β-NAD^+, 0.2 mmol of potassium phosphate (pH 8.0), and an appropriate amount of enzyme in a final volume of 1.0 ml. The formation of NADH was followed at 30° by measuring the absorbance at 340 nm [6].

The deaminase activity was assayed by measuring the rate of NH_3 formation by coupling with glutamate dehydrogenase (EC 1.4.1.4, GLDH) as follows. The standard mixture contained 30 μmol of

N^{α}-Z-D-lysine, 0.36 μmol of β-NADPH, 20 units of GLDH, 3 μmol of 2-oxoglutaric acid, 0.1 mmol of potassium phosphate (pH 7.5), and an appropriate amount of enzyme in a final volume of 1.0 ml. The amount of $NADP^+$ generated from NH_3 was spectrophotometrically followed at 30° by measuring the absorbance at 340 nm.

One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one μmol of H_2O_2, NADH, or NADPH per min, resp.

REFERENCES

[1] T. Hammer, R. Bode, *Zentralbl. Mikrobiol*. **1992**, *147*, 65.
[2] T. Fujii, T. Narita, H. Agematu, N. Agata, K. Isshiki, *J. Biochem*. **2000**, *128*, 391.
[3] K. Isobe, K. Tokuta, Y. Narita, A. Matsuura, T. Sakaguchi, N. Wakao, *J. Mol. Catal. B: Enzym.* **2004**, *32*, 27.
[4] K. Isobe, S. Nagasawa, K. Tokuta, A. Matsuura, T. Sakaguchi, N. Wakao, *J. Biosci. Bioeng*. **2005**, *100*, 288.
[5] K. Isobe, S. Nagasawa, *J. Biosci. Bioeng*. **2007**, *104*, 218.
[6] K. Isobe, N. Fukuda, S. Nagasawa, *J. Biosci. Bioeng*. **2007**, *104*, 398.
[7] K. Isobe, K. Tokuta, Y. Narita, A. Matsuura, T. Sakaguchi, N. Wakao, *J. Mol. Catal. B: Enzym.* **2004**, *30*, 119.
[8] K. Isobe, K. Tokuta, Y. Narita, A. Matsuura, T. Sakaguchi, N. Wakao, *J. Mol. Catal. B: Enzym.* **2004**, *30*, 13.
[9] M. A. Paz, O. O. Blumenfeld, M. Rojkind, E. Henson, C. Furfine, P. M. Gallop, *Arch. Biochem. Biophys.* **1965**, *109*, 548.