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Analysis of Selective Production of N^{α}-Benzyloxycarbonyl-L-Aminoadipate-δ-Semialdehyde and N^{α}-Benzyloxycarbonyl-L-Aminoadipic Acid by *Rhodococcus* sp. AIU Z-35-1

Kimiyasu Isobe,[1]* Nahoko Fukuda,[1] and Shouko Nagasawa[1]

Department of Agro-bioscience, Faculty of Agriculture, Iwate University, 3 Ueda, Morioka 020-8550, Japan[1]

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The factors for selective production of N^{α}-benzyloxycarbonyl-L-aminoadipate-δ-semialdehyde (N^{α}-Z-L-AASA) and N^{α}-benzyloxycarbonyl-L-aminoadipic acid (N^{α}-Z-L-AAA) from N^{α}-benzyloxycarbonyl-L-lysine (N^{α}-Z-L-lysine) by *Rhodococcus* sp. AIU Z-35-1 were analyzed. The cultivation time was important for selective production of N^{α}-Z-L-AASA, since N^{α}-Z-L-lysine oxidizing enzyme reached maximum at the early stage of cell growth and then decreased. The differences of enzyme activities of N^{α}-Z-L-lysine oxidizing enzyme and N^{α}-Z-L-AASA dehydrogenase in pH and temperature also affected the selective production of N^{α}-Z-L-AASA. For efficient production of N^{α}-Z-L-AAA, it was important for cultivation time that N^{α}-Z-L-AASA dehydrogenase activity be higher than N^{α}-Z-L-lysine oxidizing enzyme activity, since a high concentration of N^{α}-Z-L-AASA inhibited N^{α}-Z-L-AASA dehydrogenase activity. The optimum pH of N^{α}-Z-L-AAA production was affected by the instability of N^{α}-Z-L-AASA dehydrogenase in the alkaline pH region.

[**Key words:** N^{α}-benzyloxycarbonyl-L-lysine, N^{α}-benzyloxycarbonyl-L-aminoadipate-δ-semialdehyde, N^{α}-benzyloxycarbonyl-L-aminoadipic acid, L-amino acid oxidase, aldehyde dehydrogenase]

L-α-Aminoadipate-δ-semialdehyde (L-α-AASA) and L-α-aminoadipic acid (L-α-AAA) are important precursors in biosynthesis of β-lactam antibiotics (1), and those and the related compounds are useful raw materials for chemical synthesis of new antibiotics or physiologically active peptides. The biochemical methods for the production of L-α-AASA, L-α-AAA, and their derivatives have been reported, but they were complicated and their conversion yields were low (2–5). To overcome such drawbacks, we recently isolated a new bacterial strain, *Rhodococcus* sp. AIU Z-35-1, as a high producer of N^{α}-Z-L-AAA, in which N^{α}-benzyloxycarbonyl-L-lysine (N^{α}-Z-L-lysine) was converted into N^{α}-Z-L-AAA via N^{α}-Z-L-AASA as shown in Fig. 1 (6). We have then developed the improved methods for production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA using a new isolated strain. Thus, N^{α}-Z-L-AAA was efficiently produced by incubating at pH 7.0 and 30°C with cells harvested after 3 d of cultivation (6). The selective production of N^{α}-Z-L-AASA was achieved by incubating at pH 5.0 and 30°C with cells harvested after 1 d of cultivation. The N^{α}-Z-L-AASA was also produced with high conversion yields by incubation at pH 7.0 and 10°C with cells harvested after 1 d of cultivation (7). These methods were superior to other biochemical methods, but the reasons for selective production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA had not been clarified. More recently, we revealed that the conversion of N^{α}-Z-L-lysine to N^{α}-Z-L-AASA was catalyzed by an L-amino acid oxidase with broad substrate specificity (8), and that of N^{α}-Z-L-AASA to N^{α}-Z-L-AAA was catalyzed by an aldehyde dehydrogenase (9). In the analyses of enzymatic properties of both enzymes, it was revealed that both oxidations of N^{α}-Z-L-lysine by an L-amino acid oxidase and of N^{α}-Z-L-AASA by an aldehyde dehydrogenase were optimum at around pH 8.0. Thus, optimum pHs of both enzyme activities were significantly different from those of the production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA by cell reaction. Therefore, the factors involved in the selective production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA by cell reaction are analyzed in detail in this report.

MATERIALS AND METHODS

Chemicals N^{α}-Z-L-AASA was prepared according to the method described in our previous report (7). N^{α}-Z-L-Lysine and nitro blue tetrazolium were obtained from Watanabe Chemical Industries (Hiroshima) and Sigma Chemical Japan (Tokyo), respectively, and β-NAD^{+} and β-$NADP^{+}$ were from Oriental Yeast (Osaka). Peroxidase and diaphorase were gifts from Amano Enzyme (Nagoya). All other chemicals used were of analytical grade and commercially available.

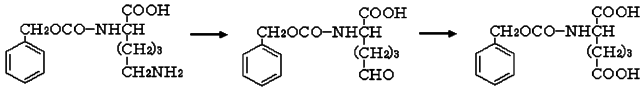

FIG. 1. Conversion of N^{α}-Z-L-lysine into N^{α}-Z-L-AAA by *Rhodococcus* sp. AIU Z-35-1.

* Corresponding author. e-mail: kiso@iwate-u.ac.jp
phone/fax: +81-(0)19-621-6155

Cultivation and enzyme preparation *Rhodococcus* sp. AIU Z-35-1 was incubated in the N^{α}-Z-L-lysine medium or L-lysine medium, pH 7.0, consisting of 0.2% KH_2PO_4, 0.1% Na_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% glucose, and 0.5% N^{α}-Z-L-lysine or 0.5% L-lysine, respectively (8). The cells were harvested by centrifugation at 20,000×*g* for 10 min, washed with 50 mM potassium phosphate buffer, pH 7.0, and then stored at −20°C until use. The cell-free extract was prepared by disrupting the cells with glass beads using a Multi-beads shocker (Yasui Kikai, Osaka). L-Amino acid oxidase and N^{α}-Z-L-AASA dehydrogenase were purified according to the procedure of our reports (8, 9). The enzymatic and kinetic analyses of both enzymes were carried out using the purified L-amino acid oxidase with specific activity of 19.1 units per mg of protein and the N^{α}-Z-L-AASA dehydrogenase with specific activity of 1.44 units per mg of protein.

Assay of oxidase activity Oxidase activity was assayed by measuring the formation rate of hydrogen peroxide as follows. The standard reaction mixture contained 40 μmol of N^{α}-Z-L-lysine, 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 formation of hydrogen peroxide was spectrophotometrically followed at 30°C for 5 min by measuring the absorbance at 555 nm, and the enzyme activity was calculated using molar absorption coefficient of 16,500 M^{-1} cm^{-1} for the dye formed. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole of hydrogen peroxide per min under the above conditions.

Assay of dehydrogenase activity The dehydrogenase activity was assayed by measuring the formation rate of NADH as follows. The standard reaction mixture contained 20 μmol of N^{α}-Z-L-AASA and 0.6 μmol of β-NAD^+, 0.2 mmol of potassium phosphate, pH 7.0, and an appropriate amount of enzyme in a final volume of 1.0 ml. The formation of NADH was followed at 30°C by measuring the absorbance at 340 nm, and the enzyme activity was calculated using molar absorption coefficient of 6220 M^{-1} cm^{-1} for NADH. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole of NADH per min.

Standard reaction of N^{α}-Z-L-AAA production The standard reaction mixture was composed of 100 mM N^{α}-Z-L-lysine, 0.1 M potassium phosphate buffer, pH 7.0, and the bacterial cells from 100 ml culture broth, in a final volume of 1.5 ml. The reaction mixture was pipetted into a test tube (13×1.5 cm of diameter), and incubated at 30°C for 6 d with shaking at 120 strokes per min. The reaction was terminated by separating the cells by centrifugation at 20,000×*g* for 5 min, and the supernatant was used for assay of the reaction products.

Analysis of reaction products The reaction products from N^{α}-Z-L-lysine were analyzed by HPLC with a TSK-Gel DEAE-5PW column under the same conditions as Isobe *et al.* (6), and each concentration was calculated by the peak area.

RESULTS

Effects of cultivation time on enzyme production

Rhodococcus sp. AIU Z-35-1 reportedly produced an L-amino acid oxidase exhibiting N^{α}-Z-L-lysine oxidase activity and an aldehyde dehydrogenase exhibiting N^{α}-Z-L-AASA dehydrogenase activity by incubating in a medium containing N^{α}-Z-L-lysine or L-lysine (8, 9), but the optimum cultivation time for the production of both enzymes has not been studied. Therefore, this strain was incubated in the medium containing 0.5% N^{α}-Z-L-lysine or 0.5% L-lysine at 30°C for 5 d, and both enzyme activities were assayed each day using cell-free extract. The N^{α}-Z-L-lysine oxidase activity reached maximum at one day of cultivation in both media, but the maximum product amounts of this oxidase in the L-lysine medium was 3.5 times higher than that in the N^{α}-Z-L-lysine medium. The oxidase amount was then reduced by a long-time cultivation in both media, but the reducing speed in the L-lysine medium was faster than that in the N^{α}-Z-L-lysine medium. In contrast, the N^{α}-Z-L-AASA dehydrogenase activity reached maximum at 3 d of cultivation in both media, and its maximum product amounts in the L-lysine medium were three times higher than that in the N^{α}-Z-L-lysine medium (Fig. 2). These results indicate that cells from the L-lysine

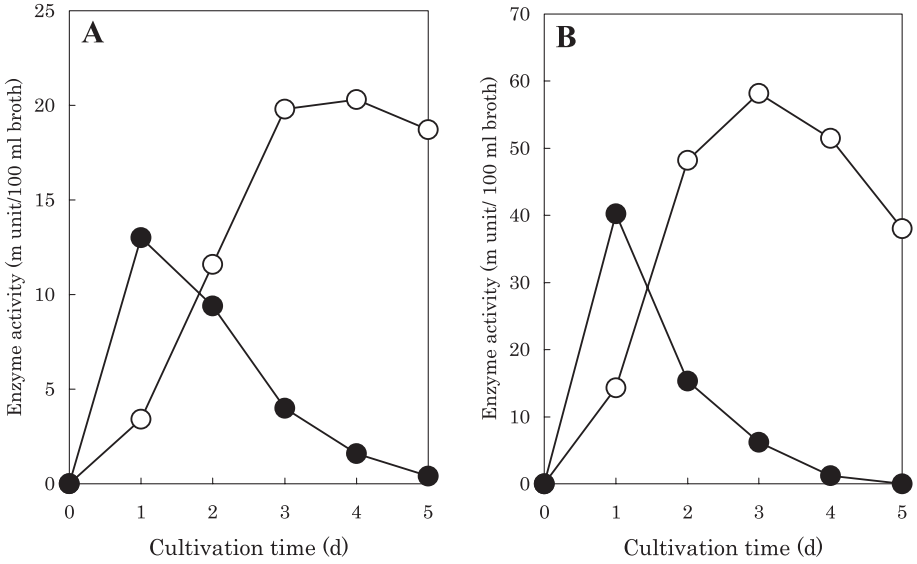

FIG. 2. Effects of cultivation time on production of N^{α}-Z-L-lysine oxidase and N^{α}-Z-L-AASA dehydrogenase. *Rhodococcus* sp. AIU Z-35-1 was incubated in the N^{α}-Z-L-lysine medium (A) or the L-lysine medium (B) at 30°C for 5 d, and N^{α}-Z-L-lysine oxidase activity (closed circles) and N^{α}-Z-L-AASA dehydrogenase activity (open circles) were assayed using cell-free extract each day under standard assay conditions.