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New biochemical methods for production of N^{α}-benzyloxycarbonyl-D,L-aminoadipic-δ-semialdehyde and N^{α}-benzyloxycarbonyl-D,L-aminoadipic acid

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Abstract

New biochemical methods for the production of N^{α}-benzyloxycarbonyl-L-aminoadipate δ-semialdehyde (N^{α}-Z-L-AASA), N^{α}-Z-D-AASA, N^{α}-Z-L-aminoadipic acid (N^{α}-Z-L-AAA), N^{α}-Z-D-AAA were developed using cells of *Rhodococcus* sp. AIU Z-35-1. When the cells harvested after 1 day of cultivation were incubated with 100 mM N^{α}-Z-L-lysine at pH 5.0 for 1 day at 30 °C or at pH 7.0 for 2 days at 10 °C, more than 95 mM N^{α}-Z-L-AASA was produced. Using the cells harvested after 3 days of cultivation, N^{α}-Z-L-AAA was efficiently produced by incubating at pH 7.0 for 4 days at 30 °C. Similar conversion yields of N^{α}-Z-D-AASA and N^{α}-Z-D-AAA were also obtained under the same conditions. Thus, the microbial methods proposed here were superior to the chemical and other biochemical methods in simplicity, reaction rate, and yield.

Keywords: N^{α}-Z-L-lysine; N^{α}-Z-D-lysine; N^{α}-Z-L-aminoadipate δ-semialdehyde; N^{α}-Z-D-aminoadipate δ-semialdehyde; N^{α}-Z-L-aminoadipic acid; N^{α}-Z-D-aminoadipic acid

1. Introduction

L-α-Aminoadipic acid (L-α-AAA) is a precursor of β-lactam antibiotics, and L-α-AAA and its related compounds provide interesting raw materials for the chemical synthesis of new antibiotics or physiological peptides. We have recently developed new biochemical methods for the production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA from N^{α}-Z-L-lysine utilizing amine oxidase and mycelia from *Aspergillus niger* AKU 3302, respectively [1,2]. However, the yield of N^{α}-Z-L-AAA was not high, and little N^{α}-Z-D-AAA was formed by the method with mycelia of *A. niger*. More recently we isolated a new bacterial strain, *Rhodococcus* sp. AIU Z-35-1, capable of producing N^{α}-Z-L-AAA and N^{α}-Z-D-AAA with a high conversion yield [3]. Since this strain formed N^{α}-Z-L-AASA in the conversion of N^{α}-Z-L-lysine into N^{α}-Z-L-AAA, we investigated the efficient methods for the production of N^{α}-Z-L-AAA, N^{α}-Z-D-AAA, N^{α}-Z-L-AASA and N^{α}-Z-D-AASA using the new isolated strain.

2. Materials and methods

2.1. Cultivation

Rhodoccocus sp. Z-35-1 was first cultured in a test tube containing 5 ml of the N^{α}-Z-L-lysine medium, pH 7.0, which consisted 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, at 30 °C for 2 days with shaking at 120 strokes/min. The culture (1.5 ml) was then inoculated into a 500-ml shaker flask containing 150 ml of the same medium. The second culture was incubated at 30 °C for 1–3 days with shaking at 120 strokes/min. Cells were harvested by centrifugation at 20,000 × *g* for 10 min, washed with 0.1 M potassium phosphate buffer, pH 7.0, and stored at −20 °C until use.

2.2. Standard reaction

Seventy-five micromoles of N^{α}-Z-L-lysine or N^{α}-Z-D-lysine was incubated with cells from 50 to 200 ml of culture broth at 30 °C for 1–6 days in 1.5 ml of 0.1 M potassium phosphate buffer, pH 7.0, with shaking at 120 strokes/min. The reaction was terminated by separating the cells by centrifugation at 20,000 × *g* for 5 min, and the supernatant was used for the assay of reaction products.

2.3. Analysis of reaction products

The amounts of reaction products were calculated from the peak area of HPLC chromatograms from a TSK-Gel DEAE-5PW column (Tosoh, Tokyo, Japan) under the conditions described in our previous report [3].

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3. Results

3.1. Identification of reaction products

Rhodococcus sp. AIU Z-35-1 was cultured in the N^{α}-Z-L-lysine medium for 3 days, and the cells from 100 ml culture broth were incubated with 20 mM N^{α}-Z-L-lysine at 30 °C for 4 days, and the reaction products were analyzed by HPLC with a TSK-Gel DEAE-5PW column. The peak of N^{α}-Z-L-lysine (elution at 3.4 min) remarkably decreased, and two new peaks appeared at 21.4 and 24.1 min, which were the same as the elution time of N^{α}-Z-L-AAA and N^{α}-Z-L-AASA, respectively [3]. The product eluted at 21.4 min was also detected at the same elution time of N^{α}-Z-L-AAA by a Syn Pro Pep C_{18} column (elution at 32.8 min). In TLC analysis of the solution eluted at 21.4 min, one spot of violet color was obtained by spraying a ninhydrin reagent after the TLC plate was treated with a hydrogen bromide–acetic acid solution, and its mobility was the same as that of N^{α}-Z-L-AAA. Another product eluted at 24.1 min was also detected at the same elution time of N^{α}-Z-L-AASA by a Syn Pro Pep C_{18} column, and it exhibited adsorption maxima at around 620 and 660 nm by the reaction with 3-methyl-2-benzothiazolinone hydrazone, indicating that this product contained an aldehyde group. When the reaction products from N^{α}-Z-D-lysine were analyzed using the same methods, N^{α}-Z-D-AASA and N^{α}-Z-D-AAA were identified. Thus, it was concluded that this new isolated strain named *Rhodococcus* sp. AIU Z-35-1 converted N^{α}-Z-L-lysine and N^{α}-Z-D-lysine into N^{α}-Z-L-AAA and N^{α}-Z-D-AAA *via* N^{α}-Z-L-AASA and N^{α}-Z-D-AASA, respectively, according to Scheme 1.

3.2. Effects of reaction conditions on production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA

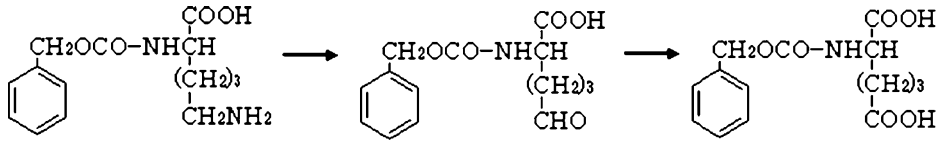
When cells from 100 ml culture broth harvested after 3 days of cultivation were incubated with 30 mM N^{α}-Z-L-lysine at pH 7.0 for 2 days, the product amount of N^{α}-Z-L-AASA was higher than that of N^{α}-Z-L-AAA, and the amount of N^{α}-Z-L-AAA gradually increased by prolonging the reaction time. These results indicated that the formation of N^{α}-Z-L-AAA was limited by the conversion rate of N^{α}-Z-L-AASA to N^{α}-Z-L-AAA, and that this strain has a possibility of selective production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA. We, therefore, investigated the reaction pH and temperature for the production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA using the cells harvested at 3 days of cultivation. When the reaction pH was varied between pH 5.0 and 8.5, the highest formation of N^{α}-Z-L-AAA was obtained at pH 7.0, and that of N^{α}-Z-L-AASA was at pH 5.0. When the reaction temperature was varied between 10 and 50 °C at pH 7.0, the maximum production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA was obtained at 10 and 30 °C, respectively. In the reaction at pH 5.0, N^{α}-Z-L-AASA was quickly produced at 30 °C. Thus, the reactions at pH 7.0 and 10 °C or at pH 5.0 and 30 °C were suitable for the production of N^{α}-Z-L-AASA, and that at pH 7.0 and 30 °C was optimum for the production of N^{α}-Z-L-AAA.

The cell amounts also affected the selective production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA. When cells from 50 to 400 ml of culture broth were incubated with 50 mM N^{α}-Z-L-lysine at pH 7.0, the product amount of N^{α}-Z-L-AASA decreased with increasing cell amounts, and that of N^{α}-Z-L-AAA increased. Thus, the product amount of N^{α}-Z-L-AAA reached a maximum with cells from 200 ml of culture broth. On the other hand, the highest conversion yield of N^{α}-Z-L-AASA was obtained using cells from 50 ml of culture broth.

The reaction time was also an important factor for the selective production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA. In the above reactions, the product amount of N^{α}-Z-L-AAA increased by prolonging the reaction time, while that of N^{α}-Z-L-AASA decreased. Thus, 1 or 2 days of reaction were suitable for the production of N^{α}-Z-L-AASA. The product amount of N^{α}-Z-L-AAA reached a maximum by incubating for 4 or 6 days.

3.3. Effects of culturing time on production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA

Although the reaction conditions for the production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA were optimized, the selective production of both compounds was not achieved. Therefore, effects of culturing time on the production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA were also investigated. When cells from 50 ml of culture broth harvested after 1 day of cultivation were incubated with 80 mM N^{α}-Z-L-lysine at pH 5.0 and 30 °C, N^{α}-Z-L-lysine was completely consumed, and 76 mM N^{α}-Z-L-AASA and 4 mM N^{α}-Z-L-AAA were formed. When cells harvested after more than 2 days of cultivation were used, the product amount of N^{α}-Z-L-AAA was larger than that of the cells harvested after 1 day of cultivation, while the consumption rate of N^{α}-Z-L-lysine was reduced. These results indicated that cells harvested after 1 day of cultivation were suitable for the production of N^{α}-Z-L-AASA, and cells harvested after 2 or 3 days of cultivation were effective for the production of N^{α}-Z-L-AAA.



Scheme 1. Conversion of N^{α}-Z-L-lysine and N^{α}-Z-D-lysine by reaction with cells from *Rhodococcus* sp. AIU Z-35-1.

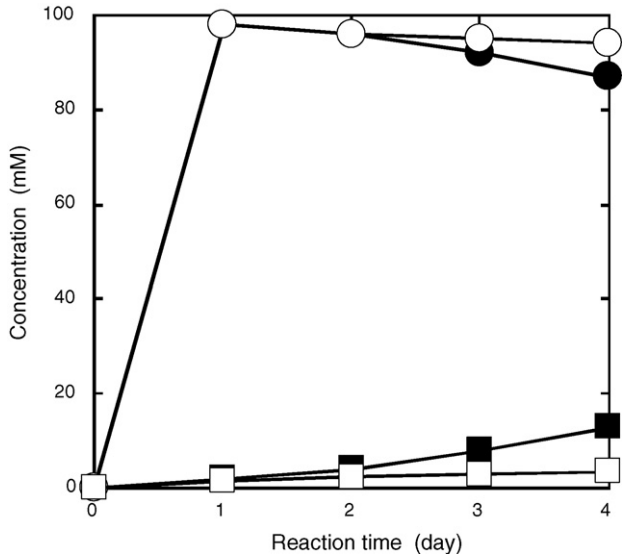


Fig. 1. Production of N^{α}-Z-L-AASA and N^{α}-Z-D-AASA at pH 5.0. About 100 mM N^{α}-Z-L-lysine or N^{α}-Z-D-lysine was incubated at pH 5.0 for 4 days at 30 °C with cells from 50 ml of culture broth harvested at 1 day of cultivation. Closed circles, N^{α}-Z-L-AASA; open circles, N^{α}-Z-D-AASA; closed squares, N^{α}-Z-L-AAA; open squares, N^{α}-Z-D-AAA.

3.4. Production of N^{α}-Z-L-AASA and N^{α}-Z-D-AASA

When cells from 50 ml of culture broth harvested after 1 day of cultivation were incubated with 100 mM N^{α}-Z-L-lysine at pH 5.0 for 1 day at 30 °C, more than 95 mM N^{α}-Z-L-AASA was produced (Fig. 1). When this reaction was carried out at pH 7.0 for 2 days at 10 °C, N^{α}-Z-L-AASA was also produced with similar conversion yield (Fig. 2). The production of N^{α}-Z-D-AASA was also investigated using 100 mM N^{α}-Z-D-lysine under the above conditions. More than 95 mM N^{α}-Z-D-AASA

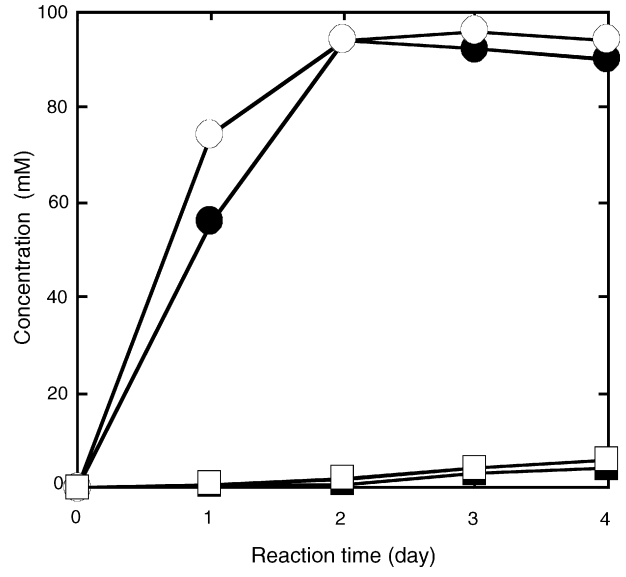


Fig. 2. Production of N^{α}-Z-L-AASA and N^{α}-Z-D-AASA at pH 7.0. About 100 mM N^{α}-Z-L-lysine or N^{α}-Z-D-lysine was incubated at pH 7.0 for 4 days at 10 °C with cells from 50 ml of culture broth harvested at 1 day of cultivation. Closed circles, N^{α}-Z-L-AASA; open circles, N^{α}-Z-D-AASA; closed squares, N^{α}-Z-L-AAA; open squares, N^{α}-Z-D-AAA.

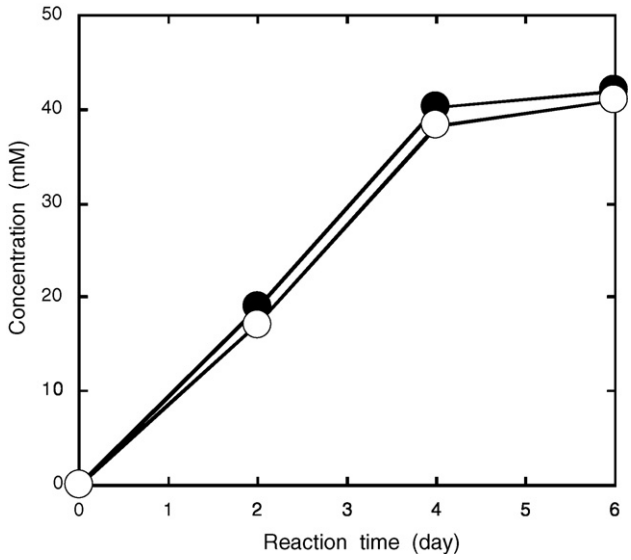


Fig. 3. Production of N^{α}-Z-L-AAA and N^{α}-Z-D-AAA at pH 7.0. About 50 mM N^{α}-Z-L-lysine or N^{α}-Z-D-lysine was incubated at pH 7.0 for 4 days at 30 °C with cells from 200 ml of culture broth harvested at 3 days of cultivation. Closed circles, N^{α}-Z-L-AAA; open circles, N^{α}-Z-D-AAA.

was also produced by incubating at pH 5.0 for 1 day at 30 °C or at pH 7.0 for 2 days at 10 °C. These reaction times were two or four times shorter than that of the amine oxidase method, and the yields of N^{α}-Z-L-AASA and N^{α}-Z-D-AASA were two times higher than those of the amine oxidase method.

3.5. Production of N^{α}-Z-L-AAA and N^{α}-Z-D-AAA

When cells from 200 ml of culture broth harvested at 3 days of cultivation were incubated with 50 mM N^{α}-Z-L-lysine at pH 7.0 for 4 days at 30 °C, approximately 40 mM N^{α}-Z-L-AAA was produced (Fig. 3). Thus, a more than 10-fold higher concentration of N^{α}-Z-L-AAA was produced in one-third of the reaction time of *A. niger*. Using 50 mM N^{α}-Z-D-lysine, production of N^{α}-Z-D-AAA was investigated under the same conditions. N^{α}-Z-D-AAA was also produced with a similar conversion yield.

4. Discussion

Although *Rhodococcus* sp. AIU Z-35-1 had a high ability of the conversion of N^{α}-Z-L-lysine into N^{α}-Z-L-AAA, the conversion of N^{α}-Z-L-lysine to N^{α}-Z-L-AASA was faster than that of N^{α}-Z-L-AASA to N^{α}-Z-L-AAA. We therefore investigated efficient methods for the selective production of N^{α}-Z-L-AASA and N^{α}-Z-L-AAA using this microorganism [3,4]. In those developments, a combination of the reaction conditions and culturing time was important, since the conversion of N^{α}-Z-L-lysine into N^{α}-Z-L-AAA was catalyzed by two enzymes, and both enzymes differed from one another in their optimum reaction conditions and production time. The methods for the production of N^{α}-Z-L-AASA were developed by obtaining high activity of the conversion of N^{α}-Z-L-lysine into N^{α}-Z-L-AASA and by reducing the conversion of N^{α}-Z-L-AASA into N^{α}-Z-L-AAA. On the other hand, the method for the production of N^{α}-Z-L-AAA was

developed by obtaining high activity of two enzymes catalyzing the conversion of N^{α}-*Z*-L-lysine into N^{α}-*Z*-L-AASA and N^{α}-*Z*-L-AASA into N^{α}-*Z*-L-AAA. Thus, N^{α}-*Z*-L-AASA was produced with a conversion yield of more than 95% from 100 mM N^{α}-*Z*-L-lysine by incubating at pH 5.0 and 30 °C for 1 day or at pH 7.0 and 10 °C for 2 days with cells from 50 ml of culture broth harvested at 1 day of cultivation. N^{α}-*Z*-L-AAA was produced with a conversion yield of more than 80% from 50 mM N^{α}-*Z*-L-lysine by incubating at pH 7.0 and 30 °C for 4 days with cells from 200 ml of culture broth harvested at 3 days of cultivation. These methods were markedly superior to the other methods in simplicity, reaction rate, and yield. In addition, these conditions were applicable for the efficient production of N^{α}-*Z*-D-AASA and N^{α}-*Z*-D-AAA. Thus, the microbial methods proposed here were useful for the production of N^{α}-*Z*-L-AASA, N^{α}-*Z*-D-AASA, N^{α}-*Z*-L-AAA and N^{α}-*Z*-D-AAA.

References

[1] Isobe K, Tokuta K, Narita Y, Matsuura A, Sakaguchi T, Wakao N. A method for production of N^{α}-benzyloxycarbonyl-aminoadipate-δ-semialdehyde with amine oxidase from *Aspergillus niger*. J Mol Catal B: Enzym 2004;30:119–23.

[2] Isobe K, Tokuta K, Narita Y, Matsuura A, Sakaguchi T, Wakao N. Formation of N^{α}-benzyloxycarbonyl-L-aminoadipic acid from N^{α}-benzyloxycarbonyl-L-lysine with mycelia from *Aspergillus niger*. J Mol Catal B: Enzym 2004;30:13–8.

[3] Isobe K, Tokuta K, Narita Y, Matsuura A, Sakaguchi T, Wakao N. Production of N^{α}-benzyloxycarbonyl-L-aminoadipic acid and N^{α}-benzyloxycarbonyl-D-aminoadipic acid with *Rhodococcus* sp. AIU Z-35-1. J Mol Catal B: Enzym 2004;32:27–32.

[4] Isobe K, Nagasawa S, Tokuta K, Narita Y, Matsuura A, Sakaguchi T, et al. A new microbial method for more efficient production of N^{α}-benzyloxycarbonyl-L-aminoadipate-δ-semialdehyde and N^{α}-benzyloxycarbonyl-D-aminoadipate-δ-semialdehyde. J Biosci Bioeng 2005;100:288–91.