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Formation of N^{α}-benzyloxycarbonyl-L-aminoadipic acid from N^{α}-benzyloxycarbonyl-L-lysine with mycelia from *Aspergillus niger*

Kimiyasu Isobe [a,*], Keigo Tokuta [a], Yuuki Narita [a], Akira Matsuura [b], Takehiko Sakaguchi [b], Norio Wakao [a]

[a] *Department of Agro-bioscience, Faculty of Agriculture, Iwate University, 3 Ueda, Morioka 020-8550, Japan*
[b] *Sanyo Fine Co. Ltd., 1 Hiranomachi, Chuo-ku, Osaka 541-0046, Japan*

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Abstract

A method for formation of N^{α}-benzyloxycarbonyl-L-aminoadipic acid (N^{α}-*Z*-L-AAA) was investigated. N^{α}-*Z*-L-AAA was formed from N^{α}-*Z*-L-lysine via N^{α}-*Z*-L-aminoadipate-δ-semialdehyde (N^{α}-*Z*-L-AASA) by the reaction with mycelia from *Aspergillus niger* according to Scheme 1. In this reaction, oxidation of N^{α}-*Z*-L-lysine to N^{α}-*Z*-L-AASA was catalyzed by amine oxidase. The reaction at pH 7.0 and 30 °C was optimum for N^{α}-*Z*-L-AAA formation, and approximately 50% of the decomposed N^{α}-*Z*-L-lysine was converted into N^{α}-*Z*-L-AAA.

Keywords: Amine oxidase; *Aspergillus niger*; N^{α}-Z-L-lysine; N^{α}-Z-L-aminoadipate; N^{α}-Z-L-aminoadipate-δ-semialdehyde

1. Introduction

L-α-Aminoadipic acid (L-α-AAA) is an intermediate in the metabolism of L-lysine and a component of natural β-lactam antibiotics [1–3]. Recently, L-α-AAA and related compounds were noted to be raw materials for chemical synthesis of new antibiotics or physiological peptides, and biochemical methods for L-α-AAA production were developed using L-lysine or L-α-ketoadipate as a starting material [4,5]. In the L-lysine method, L-lysine was converted into L-α-AAA by a two-step reaction; in the first step, it was converted into L-α-aminoadipate-δ- semialdehyde (L-α-AASA) by lysine-6-aminotransferase [6] or lysine-ε- dehydrogenase [7]. The resulting product was non-enzymatically cyclized to form Δ-1-piperideine-6-carboxylate, and then converted into L-α-AAA by Δ-1-piperideine-6-carboxylate dehydrogenase [4,8]. Since L-α-AAA derivatives are also useful as raw materials for chemical synthesis of new antibiotics or physiological peptides, we studied a microbial method for production of L-α-AAA derivatives, and revealed that N^{α}-*Z*-L-AAA was formed from N^{α}-Z-L-lysine by the reaction with mycelia from *Aspergillus niger*. The present paper describes identification of the reaction products from N^{α}-*Z*-L-lysine, together with the optimum conditions for production of N^{α}-*Z*-L-AAA.

2. Materials and methods

2.1. Chemicals

L-Lysine and L-AAA were purchased from Wako Pure Chemicals (Osaka, Japan), and N^{α}-*Z*-L-lysine was purchased from Calbiochem–Novabiochem (Läufelfingen, Switzerland). N^{α}-*Z*-L-AAA was obtained from Sanyo Fine (Osaka, Japan). *A. niger* AKU 3302 was the kind gift of professor S. Shimizu, Kyoto University (Laboratory of Applied Microbiology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan). Crystalline amine oxidase from *A. niger* was kindly provided by professor O. Adachi, Yamaguchi University (Yamaguchi, Japan). All other chemicals used were of analytical grade and commercially available.

Abbreviations: N^{α}-Z-L-lysine, N^{α}-benzyloxycarbonyl-L-lysine; MBTH, 3-methyl-2-benzothiazolinone hydrazone; TOOS, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dihydrate

*Corresponding author. Tel.: +81-19-621-6155; fax: +81-19-621-6155.
E-mail address: kiso@iwate-u.ac.jp (K. Isobe).

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2.2. Preparation of lyophilized mycelia and cell-free extract from A. niger

A. niger AKU 3302 was cultured according to the method of Yamada et al. [9] at 30 °C for 2 days in *n*-butylamine medium. After mycelia were harvested by filtration, they were washed with distilled water and lyophilized. The cell-free extract was prepared by disrupting the harvested mycelia with a Multi-bead shocker (Yasui Kikai, Osaka, Japan) at 2500 rpm for 4 min (2 min × 2) at 4 °C. The supernatant obtained by centrifugation at 18,000 × *g* for 10 min was used for the enzyme assay.

2.3. Standard reaction of N^{α}-Z-L-lysine with mycelia

Twenty micro molars of N^{α}-Z-L-lysine was incubated with 40 mg of lyophilized mycelia at 30 °C for 12 days in 1.5 ml of 0.1 M potassium phosphate buffer, pH 7.0, with shaking. The reaction was terminated by separating the mycelia by centrifugation at 20,000 × *g* for 5 min, and the supernatant was used for assay of the reaction products.

2.4. Analysis of reaction products

The reaction products from N^{α}-Z-L-lysine were analyzed with TLC and HPLC. The aldehyde group of the reaction product was analyzed with 3-methyl-2-benzothiazolinone hydrazone (MBTH).

2.4.1. TLC method

TLC was carried out with a Silica gel 60 plate (Merck, Darmstadt, Germany) and two solvents: solvent-1, ethanol:water (70:30), and solvent-2, phenol:water (75:25). After development of the samples, a 25% hydrogen bromide–acetic acid solution was sprayed on a TLC plate to emerge an α-amino group. The products were detected with ninhydrin reagent.

2.4.2. HPLC method

2.4.2.1. TSK-Gel DEAE-5PW column. The reaction products were separated by a TSK-Gel DEAE-5PW column (Tosoh, Tokyo, Japan) at a flow rate of 0.8 ml per min at 40 °C. The column was eluted with water for 5 min, followed by increasing the NaCl concentration to 0.3 M with a linear gradient for 10 min, and then by 0.3 M NaCl for 10 min. Detection was carried out at 210 nm.

2.4.2.2. Syn Pro Pep C_{18} column. The reaction products were separated by a Syn Pro Pep C_{18} column (Shimadzu, Kyoto, Japan) with an eluent of 0.1% trifluoroacetic acid in water for 5 min, followed by a linear gradient (0–45%) with 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile for 45 min, and then by 45% of 0.1% trifluoroacetic acid in acetonitrile for 5 min at a flow rate of 1.0 ml per min. Peaks were monitored at 210 nm.

2.4.2.3. Identification of aldehyde group. The aldehyde group of reaction products was identified with MBTH according to the method of Paz et al. [10]; the eluate from HPLC was added to 0.2 M glycine–HCl buffer, pH 4.0 (0.75 ml), and mixed with 0.3 ml of 1.0% MBTH (derivative 1). Derivative 2 was prepared by adding 0.75 ml of 0.2% $FeCl_3$ solution to 0.25 ml of derivative 1. Spectra of the derivatives were analyzed with a Shimadzu spectrophotometer UV-2450 (Kyoto, Japan).

2.5. Assay of enzyme activity for oxidation of N^{α}-Z-L-lysine and N^{α}-Z-L-AASA

The enzyme activities on the oxidation of N^{α}-Z-L-lysine and N^{α}-Z-L-AASA were spectrophotometrically assayed using the cell-free extract as follows.

2.5.1. Oxidase activity

The reaction mixture (0.8 ml) was composed of a 12.5 or 25 mM substrate and a color reagent consisting of 0.122 mg of 4-aminoantipyrine, 0.643 mg of TOOS, and 6.7 units of peroxidase per ml of 0.1 M potassium phosphate, pH 7.0. The reaction was started by the addition of 0.2 ml of enzyme solution, and the formation of hydrogen peroxide was monitored at 30 °C for 2 h by measuring the absorbance change at 550 nm.

2.5.2. Dehydrogenase activity

The reaction mixture (0.8 ml) was composed of a 12.5 or 25 mM substrate and a color reagent consisting of 0.3 mg nitroblue tetrazolium, 3.0 mM β-NAD^+ and 2 units of diaphorase in 0.1 M potassium phosphate buffer, pH 7.0. The reaction started by the addition of 0.2 ml of enzyme solution, and the NADH formation was monitored at 30 °C for 2 h by measuring the absorbance change at 550 nm.

3. Results

3.1. HPLC analysis of reaction products from N^{α}-Z-L-lysine

First, N^{α}-Z-L-lysine and its related compounds were analyzed by HPLC with the TSK-Gel DEAE-5PW column. L-Lysine, N^{α}-Z-L-lysine, L-α-AAA and N^{α}-Z-L-AAA were eluted at 2.7, 3.4, 14.8, and 21.4 min, respectively, under the conditions described in Section 2. Then, reaction products from N^{α}-Z-L-lysine were analyzed under the same conditions. When 20 mM N^{α}-Z-L-lysine was incubated with mycelia of *A. niger* at 30 °C for 2 days, the peak of N^{α}-Z-L-lysine decreased, and two peaks were newly detected at 21.4 and 24.1 min (the former peak was large, and

Table 1
Retention time and rf value of reaction products and standard amino acids

Amino acid	HPLC (min)		TLC (rf value)	
	DEAE	C_{18}	Solvent 1	Solvent 2
N^{α}-*Z*-L-lysine	3.4	27.0	0.77	0.50
N^{α}-*Z*-L-aminoadipic acid	21.4	32.8	0.92	0.35
L-α-Aminoadipic acid	14.8	2.8	0.69	0.20
L-Lysine	2.7	2.2	0.03	0.03
Reaction product 1	21.4	32.8	0.92	0.35
Reaction product 2	24.1	–	–	–

TLC was carried out with a silica gel 60 plate. Solvent 1, ethanol:water (70:30); Solvent 2, phenol:water (75:25). Products were detected with ninhydrin reagent after 25% hydrogen bromide–acetic acid solution was sprayed on TLC plate and dried. Reaction product 1 was obtained from reaction of N^{α}-*Z*-L-lysine with mycelia for 2 and 12 days. Reaction product 2 was obtained from reaction of N^{α}-*Z*-L-lysine with mycelia or amine oxidase for 2 days; –, not assayed.

the latter was small) (Table 1). In the supernatant of a 12-day reaction, the small peak of 24.1 min was undetectable, and the peak of 21.4 min became higher than that of a 2-day reaction (data not shown). Since these two peaks were presumed to be the oxidation products from N^{α}-*Z*-L-lysine, we analyzed both peaks by HPLC with a Syn Pro Pep C_{18} column and TLC.

3.2. Identification of product eluted at 21.4 min

When the solution eluted at 21.4 min from the TSK-Gel DEAE-5PW column was analyzed with a Syn Pro Pep C_{18} column, a single peak was detected at 32.8 min. This elution time was the same as that of N^{α}-*Z*-L-AAA (Table 1). The solution eluted at 21.4 min from the TSK-Gel DEAE-5PW column and the supernatant of a 12-day reaction with mycelia of *A. niger* were also analyzed by TLC. One new spot, which exhibited a violet color with ninhydrin reagent after treatment with a hydrogen bromide–acetic acid, was obtained from each sample on TLC plate, and their mobilities were the same as that of N^{α}-*Z*-L-AAA in both solvents; the rf values of 0.92 and 0.35 were obtained from both samples in solvents 1 and 2, respectively (Table 1). In addition, the solution eluted at 21.4 min did not exhibit a spectrum with an absorption maximum by the reaction with MBTH (data not shown). These results indicate that the compound eluted at 21.4 min from the TSK-Gel DEAE-5PW column was N^{α}-*Z*-L-AAA, which was a main product from N^{α}-*Z*-L-lysine by the reaction with mycelia from *A. niger*.

3.3. Identification of product eluted at 24.1 min

First, the aldehyde group in the product eluted at 24.1 min was analyzed with MBTH. Derivative 2 with MBTH showed two adsorption maxima at around 620 and 660 nm (Fig. 1), indicating that this product contained an aldehyde group. The solution eluted at 24.1 min turned violet with ninhydrin reagent on the TLC plate with hydrogen

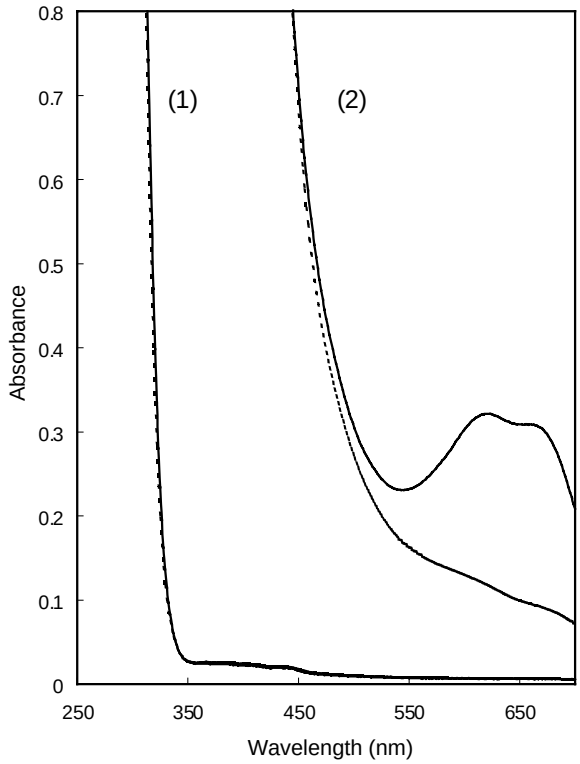
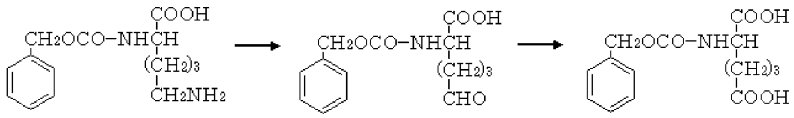


Fig. 1. Spectra for MBTH-derivatives 1 and 2 of a solution eluted at 24.1 min from TSK-Gel DEAE-5PW column. Derivatives 1 and 2 were prepared as described in Section 2 using water (dotted lines) and peak solution of 24.1 min (solid lines). (1) Derivative 1; (2) Derivative 2.

bromide–acetic acid treatment, but did not change color without this treatment. These results indicate that the product eluted at 24.1 min contained an aldehyde group and that one amino group bound to benzyloxycarbonyl bond. The molecular mass of this product was estimated to be 279.0 by a Finnigan Mass Spectrometer LCQ Deca (Thermo Electron Co., Yokohama, Japan), which agreed with the theoretical value of N^{α}-*Z*-L-AASA. Next, 20 mM N^{α}-*Z*-L-lysine was incubated with 0.5 units of the purified amine oxidase from *A. niger* at 30 °C for 4 days, and the reaction product was analyzed. The product of this reaction was eluted at 24.1 min by HPLC with the TSK-Gel DEAE-5PW column, and its MBTH derivative 2 showed two adsorption maxima at around 620 and 660 nm (adsorption spectra were the same as in Fig. 1). This product also turned violet with ninhydrin reagent on the TLC plate with hydrogen bromide–acetic acid treatment, but did not change color without this treatment. Furthermore, lyophilized mycelia from *A. niger* were added to the reaction mixture containing the oxidation product with amine oxidase, and incubated for another 2 days. It was ascertained by HPLC with the TSK-Gel DEAE-5PW column that the peak of 24.1 min disappeared and a new peak of 21.4 min was generated by this reaction with mycelia from *A. niger* (data not shown). These results indicate that the peak of 24.1 min was N^{α}-*Z*-L-AASA, and that the aldehyde group was formed by the oxidative



N^{α}-Z-L-lysine N^{α}-Z-L-AASA N^{α}-Z-L-AAA

Scheme 1. Oxidation of N^{α}-Z-L-lysine into N^{α}-Z-L-AAA by the reaction with mycelia from *A. niger*.

Table 2
Enzyme activity catalyzing oxidation of N^{α}-Z-L-AASA and N^{α}-Z-L-lysine

Substrate	H_2O_2 formation (nmol/h/ml)	NADH formation (nmol/h/ml)	NADPH formation (nmol/h/ml)
N^{α}-Z-L-lysine	78.9	ND	5.3
N^{α}-Z-L-AASA	43.2	ND	3.7

Enzyme activity was assayed with cell-free extract under standard assay conditions.

deamination of the ε-amino group of N^{α}-Z-L-lysine by amine oxidase. Thus, it was concluded that N^{α}-Z-L-lysine was converted into N^{α}-Z-L-AAA via N^{α}-Z-L-AASA by the reaction with mycelia from *A. niger* according to Scheme 1, and that the conversion of N^{α}-Z-L-lysine into N^{α}-Z-L-AASA was catalyzed by amine oxidase in mycelia.

3.4. Identification of enzymes forming N^{α}-Z-L-AAA from N^{α}-Z-L-lysine

The enzymes catalyzing the formation of N^{α}-Z-L-AAA from N^{α}-Z-L-lysine were analyzed using the cell-free extract. Although the oxidase activity was obtained on N^{α}-Z-L-lysine, NAD^+ or $NADP^+$ dependent dehydrogenase activity on N^{α}-Z-L-lysine was less than 10% of the oxidase activity, if any. These results agreed with the above data that N^{α}-Z-L-lysine was oxidized to N^{α}-Z-L-AASA by the purified amine oxidase from *A. niger*. In the enzyme responsible for the formation of N^{α}-Z-L-AAA from N^{α}-Z-L-AASA, oxidase activity on N^{α}-Z-L-AASA was also found, but NAD^+ or $NADP^+$ dependent dehydrogenase activity on N^{α}-Z-L-AASA was also less than 10% of the oxidase activity (Table 2). These results indicate that oxidation of N^{α}-Z-L-AASA into N^{α}-Z-L-AAA is mainly catalyzed by an aldehyde oxidase, and that N^{α}-Z-L-AAA is formed by a combination of two kinds of oxidase.

3.5. Optimization for N^{α}-Z-L-AAA formation

Optimum conditions for the formation of N^{α}-Z-L-AAA from N^{α}-Z-L-lysine were investigated under standard conditions for reaction of N^{α}-Z-L-lysine with mycelia, except that either the reaction pH varied between pH 6.0 and 8.0 or the reaction temperature ranged between 20 and 40 °C. The optimum pH and temperature for N^{α}-Z-L-AAA formation were obtained at pH 7.0 and 30 °C, respectively (Figs. 2 and 3). The optimum amounts of mycelia were investigated with 10–100 mg of mycelia in dry weight. The N^{α}-Z-L-AAA formation reached maximum at approximately 40 mg of dry weight and then declined upon the addition of large amounts of mycelia (data not shown). When the N^{α}-Z-L-lysine concentration varied between 5 and 100 mM, the reaction rates of N^{α}-Z-L-lysine consumption and N^{α}-Z-L-AAA formation were increased with increasing N^{α}-Z-L-lysine concentration. However, the conversion yield of N^{α}-Z-L-AAA reached maximum at 20 mM N^{α}-Z-L-lysine (data not shown). On the ba-

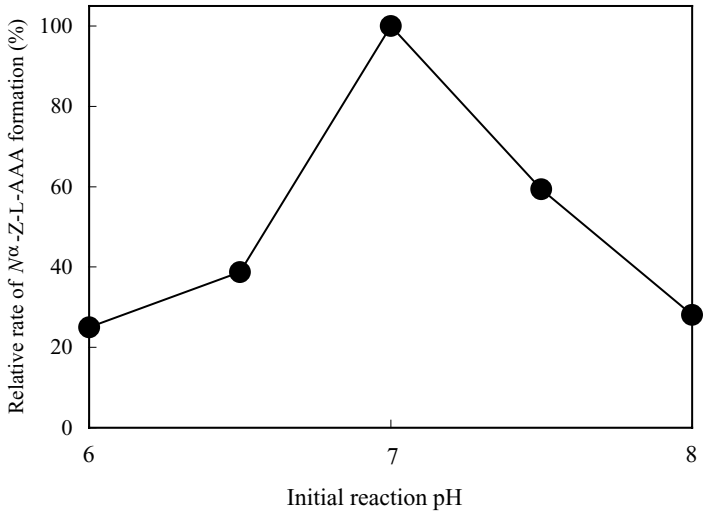


Fig. 2. Effect of pH on formation of N^{α}-Z-L-AAA from N^{α}-Z-L-lysine by the reaction with mycelia from *A. niger*. Twenty micro molars of N^{α}-Z-L-lysine was incubated with 40 mg of mycelia from *A. niger* under standard reaction conditions, except that reaction pH varied between pH 6.0 and 8.0. The concentration of N^{α}-Z-L-AAA was assayed by HPLC with a TSK-Gel DEAE-5PW column.

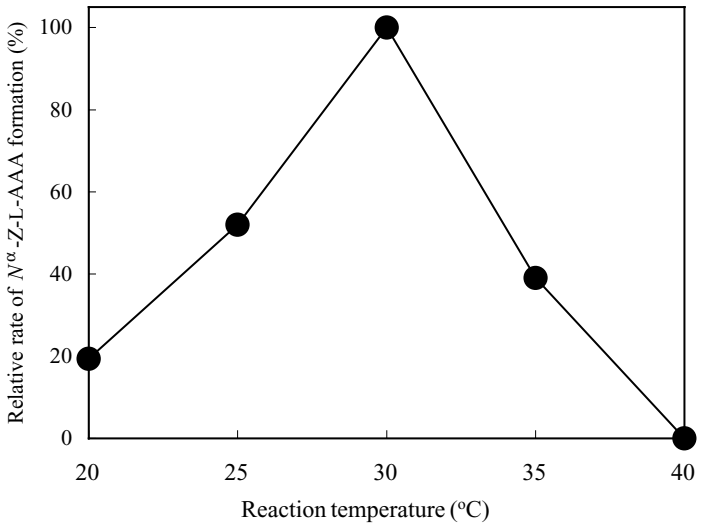


Fig. 3. Effect of reaction temperature on formation of N^{α}-Z-L-AAA from N^{α}-Z-L-lysine by the reaction with mycelia from *A. niger*. Twenty micro molars of N^{α}-Z-L-lysine was incubated with 40 mg of mycelia from *A. niger* under standard reaction conditions, except that reaction temperature was varied between 20 and 40 °C. The concentration of N^{α}-Z-L-AAA was assayed by HPLC with a TSK-Gel DEAE-5PW column.

sis of these results, 25 mM N^{α}-Z-L-lysine was incubated with 40 mg of lyophilized mycelia in 0.1 M potassium phosphate buffer, pH 7.0 at 30 °C for 12 days. Approximately 36% of the N^{α}-Z-L-lysine was consumed, and 52% of the consumed N^{α}-Z-L-lysine was accumulated as N^{α}-Z-L-AAA (Fig. 4).

4. Discussion

L-α-AAA and its related compounds have become increasingly important as raw materials for chemical synthesis of new antibiotics or functional peptides. The chemical methods for L-α-AAA synthesis have been reported, but proved to be very complicated [11]. Both enzymatic and microbial methods for L-α-AAA production have also been reported using α-ketoadipate or L-lysine as a starting material [4,5]. The method with α-ketoadipate was carried out by biphasic reaction, but turned out to be more expensive than the method with L-lysine, suggesting that the latter method may be preferable. However, the method with L-lysine required at least two reactions; L-lysine was first converted into L-α-AASA by L-lysine ε-dehydrogenase or L-lysine 6-aminotransferase, and the resulting product was then converted into L-α-AAA by Δ-1-piperideine-6-carboxylate dehydrogenase [4,8]. In these enzymatic reactions for L-α-AAA production, dehydrogenases required an additional enzyme reaction for recycling cofactors, and L-lysine 6-aminotransferase required the addition of keto acids. In

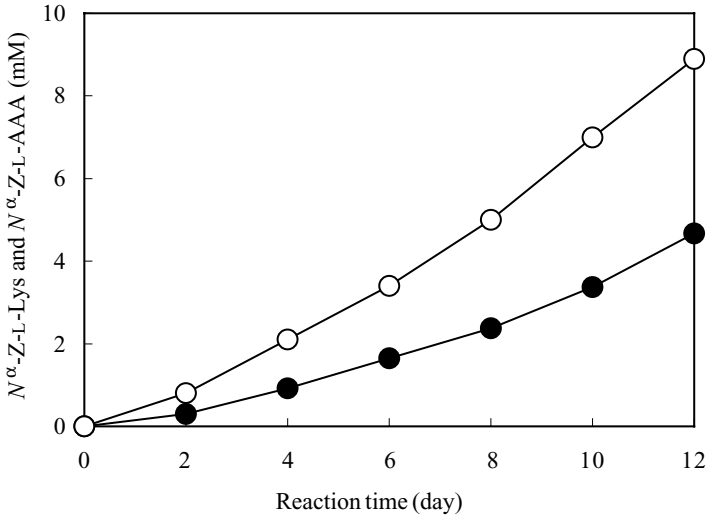


Fig. 4. Formation of N^{α}-Z-L-AAA from N^{α}-Z-L-lysine by reaction with mycelia from *A. niger*. Approximately 25 mM N^{α}-Z-L-lysine was incubated with 40 mg of mycelia of *A. niger* at pH 7.0 for 12 days at 30 °C with shaking at 120 strokes per min. Concentrations of N^{α}-Z-L-lysine consumed (open circles) and N^{α}-Z-L-AAA formed (closed circles) were assayed by HPLC with a TSK-Gel DEAE-5PW column.

general, a simple enzyme reaction with no addition of other materials except for enzymes and substrates is more desirable for efficient production of the pure product. In addition, L-lysine derivatives protecting its α-amino group are also useful for production of L-α-AAA derivatives and L-α-AAA Therefore, we attempted to develop a microbial method for the formation of N^{α}-*Z*-L-AAA from N^{α}-*Z*-L-lysine, and discovered that N^{α}-*Z*-L-lysine was converted into N^{α}-*Z*-L-AAA via N^{α}-*Z*-L-AASA by the reaction with mycelia from *A. niger* according to Scheme 1. We also revealed that oxidation of N^{α}-*Z*-L-lysine into N^{α}-*Z*-L-AASA was catalyzed by amine oxidase from *A. niger*. This amine oxidase oxidized N^{α}-*Z*-L-lysine, but not L-lysine. Yamada et al. have reported that amine oxidase from *A. niger* showed a broad substrate specificity in aliphatic and aromatic monoamines [12]. Since N^{α}-*Z*-L-lysine has the structure of a monoamine, amine oxidase might recognize it as a substrate and possibly oxidize it into N^{α}-*Z*-L-AASA. The method with mycelia from *A. niger* presented here has the advantage of requiring no cofactors or a second substrate, but the yield of N^{α}-*Z*-L-AAA was approximately 52% of the consumed N^{α}-*Z*-L-lysine. Since no peaks other than at 21.4 min were detected in the supernatant of a 12-day reaction by HPLC analysis with a TSK-Gel DEAE-5PW column, N^{α}-*Z*-L-AAA might be further decomposed by this cell-reaction. In a future study, we intend to purify the N^{α}-*Z*-L-AASA oxidizing enzyme and to increase the conversion yield of N^{α}-*Z*-L-AAA from N^{α}-*Z*-L-lysine.

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References

[1] R.A. Hartline, Methods Enzymol. 113 (1985) 639–664.
[2] J.F. Martin, Appl. Microbiol. Biotechnol. 50 (1998) 1–15.
[3] C.M. Henriksen, J. Nielsen, J. Villadsen, J. Antibiot. 51 (1998) 99–106.
[4] T. Fujita, T. Narita, H. Agematu, N. Agata, K. Isshiki, J. Biochem. 128 (2000) 975–982.
[5] J.R. Matos, C.-H. Wong, J. Org. Chem. 51 (1986) 2388–2389.
[6] T. Fujita, T. Narita, H. Agematu, N. Agata, K. Isshiki, J. Biochem. 128 (2000) 391–397.
[7] H. Misono, H. Hashimoto, H. Uehigashi, S. Nagata, S. Nagasaki, J. Biochem. 105 (1989) 1002–1008.
[8] J.L. Fuente, A. Rumbero, J.F. Martin, P. Liras, Biochem. J. 327 (1997) 59–64.
[9] H. Yamada, O. Adachi, K. Ogata, Agric. Biol. Chem. 29 (1965) 117–123.
[10] M.A. Paz, O.O. Blumenfeld, M. Rojikind, E. Henson, C. Furfine, P.M. Gallop, Arch. Biochem. Biophys. 109 (1965) 547–559.
[11] D.H.R. Barton, Y. Herve, P. Potier, J. Thierry, Tetrahedron 43 (1987) 4297–4308.
[12] H. Yamada, O. Adachi, K. Ogata, Agric. Biol. Chem. 29 (1965) 864–869.