Enantioselective *N*-Acetylation of 3-Amino-3-phenylpropionic Acid by Cell-free Extracts of *Streptomyces neyagawaensis*

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Cell-free extracts of *Streptomyces neyagawaensis* SL-387 grown on a chemically defined medium supplemented with DL-3-amino-3-phenylpropionic acid (APP) produced *N*-acetyl-APP (Ac-APP) in the presence of APP and acetyl coenzyme A. The APP obtained by acid hydrolysis of the Ac-APP was D-configuration: $[\alpha]_D+6.5°$ (H_2O) at 20°C, optical purity 92% enantiomeric excesses (ee). These results suggest that an *N*-acetyltransferase exists in the cell-free extract as a novel enzyme with specificity for D-APP.

DL-3-Amino-3-phenylpropionic acid (APP) is prepared by treating an equimolar mixture of an aromatic aldehyde and malonic acid as a pharmaceutical intermediate (1), but it is difficult to resolve the enantiomeric mixtures. In the course of studying the production of MR-387A and B by *Streptomyces neyagawaensis* SL-387, we have observed the optical resolution of DL-APP mediated by an acetyltransferase existing in cell-free extracts of the bacterium.

In a medium for the production of MR-387A and B, peptide inhibitors of aminopeptidase N (aminopeptidase M, EC 3.4.11.2) produced by *S. neyagawaensis* SL-387 (3, 4), APP was added into a chemically defined medium as an analogue of 3-amino-2-hydroxy-4-phenylbutanoic acid (AHPA) (11), a constituent amino acid of MR-387. Analysis of metabolites from the culture broth showed that *N*-acetylated APP (Ac-APP) was a major product. Although the growth of the bacterium was retarded in the chemically defined medium, production of Ac-APP was closely related to cell growth. Moreover, *N*-acetylation of APP by cell-free extracts suggests that Ac-APP is produced by an *N*-acetyltransferase with a stereospecificity for D-APP. The stereospecificity of the *N*-acetyltransferase for the substrate (D-APP) could be used for the optical resolution of DL-APP enantiomeric mixture.

N-Acetylation is catalyzed by an acetyltransferase which transfers acetyl groups from acetyl coenzyme A to the *N*-termini of amino group bearing compounds such as amino acids, proteins and antibiotics (6). The *N*-acetylation of amino acids has been extensively studied

as has been the acetylation of L-glutamate by *N*-acetylglutamate synthetase (8-10). In most cases, *N*-acetylation of a drug plays a role in its detoxification or the avoidance of self-toxicity in its producing organism (6).

In *S. neyagawaensis* SL-387, APP *N*-acetyltransferase probably plays a role in inactivation or modification of APP, or in the synthesis of a precursor of AHPA in MR-387 biosynthesis (2). However, the physiological function of *N*-acetylation of APP is unknown.

This paper deals with the optical resolution of Ac-D-APP by an *N*-acetyltransferase existing in cell-free extracts of *S. neyagawaensis* SL-387.

MATERIALS AND METHODS

Fermentation and Preparation of Cell-free Extracts

S. neyagawaensis SL-387 (3-5) was grown on 2% mannose, 0.5% ammonium acetate, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.03% $CaCl_2 \cdot 7H_2O$, 0.04% $MgSO_4 \cdot 7H_2O$, 4% (v/v) mineral solution (0.01% $FeSO_4 \cdot 7H_2O$, 0.01% $MnCl_2 \cdot 4H_2O$, and 0.01% $ZnSO_4 \cdot 7H_2O$), 10 mM DL-APP and 50 mM TES (pH 7.0) in a 5-liter jar fermentor (Korea Fermentor Co.). The strain was cultivated at 28°C for 6 days under acration (1 vvm) with agitation (250 rpm). To assay for *N*-acetyltransferase activity, mycelia were washed twice with saline and dissolved in cold 100 mM Tris·HCl buffer (pH 8.2) containing 1 mM EDTA, 1 mM dithioerythritol, 2 mM phenylmethylsulfonyl fluoride and 15% (v/v) glycerol. Mycelia (16.7 g wet weight) were disrupted ultrasonically (Branson Sonifier 450, intensity 7, total 5 min), and then centrifuged at $17,000 \times g$ for 40 min. The supernatant was brought to 50% saturation with solid ammonium sulfate and then

centrifuged. The resulting supernatant was brought to 80% saturation with ammonium sulfate. The precipitate was collected by centrifugation as described above and dissolved in 20 ml 100 mM Tris·HCl buffer (pH 8.2). The solution was desalted by passage through Sephadex G-10.

Cell-free Reaction

The reaction mixture contained, in a final volume of 200 μl: 100 mM Tris·HCl (pH 8.0), 5 mM DL-APP, 0.6 mM acetyl-CoA and crude enzyme. The reaction was started by the addition of 60 μl of enzyme to the reaction mixture and allowed to proceed for 1 h at 37°C. Then 100 μl of 0.5 N HCl were added to terminate the reaction. Ac-APP from the reaction mixture was determined by an HPLC system equipped with a Hitachi L-6200 pump and L-4000 UV detector. HPLC was conducted by detecting the reaction products at 210 nm on a YMC-ODS-A column (4.6 i.d.×250 mm) with a 15:85 mixture of acetonitrile and 0.1% trifluoroacetic acid (flow rate, 1.0 ml/min).

Preparation and Identification of Ac-APP

To obtain an authentic Ac-APP, *N*-acetylation of APP was performed in a 1:1 mixture of acetic anhydride and acetic acid at 22°C for 1 h, followed by evaporation in a centrifugal evaporator (7). Identification of Ac-APP from cell-free reaction mixtures was performed by analyzing the retention time in an HPLC chromatogram. The purified Ac-APP was then subjected to instrumental analyses such as UV, MS and NMR (¹H, ¹³C and heteronuclear multiple bond coherence, so called HMBC) in order to determine the site of acetylation. The configuration of Ac-APP was determined by measuring the optical density ($[\alpha]_D$ at 20°C) of the purified Ac-APP with a polarimeter (Schmidt & Haensch, Germany).

RESULTS AND DISCUSSION

Production of Ac-APP by Fermentation

The cell growth level of *S. neyagawaensis* SL-387 in a medium supplemented with DL-APP was up to 50% lower than that in a medium supplemented with arginine (dry weight: 12.1 g/l in arginine, 6.2 g/l in APP medium). The growth curve showed that cell growth in the APP medium was characterized by a prolonged lag phase, so that the transition from exponential to stationary phase was reached after 5 days of cultivation (Fig. 1a). To investigate the fate of APP, metabolites from the culture broth were analyzed by HPLC. As a result, a dominant product was isolated in a retention time of 12.8 min under the HPLC conditions described in Materials and Methods. The product was subjected to instrumental analyses. The molecular weight of the product was 208 (M+H) and the UV spectrum showed the characteristic peaks of the phenyl group around 260 nm. The NMR in
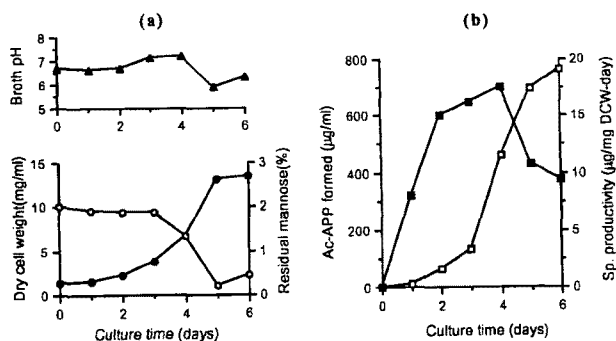

Fig. 1. Time course of cell growth and Ac-APP production in a 5-liter jar fermentor.
●, cell growth; ○, residual mannose; ▲, broth pH; □, Ac-APP formed; ■, specific productivity of Ac-APP.

CD₃OD indicates that the acetylation took place on the amino position of APP (Fig. 2). The 3-H signal underwent a downfield shift of 0.82 ppm with respect to APP; 2-H and 2'-H shifted by less than 0.08 ppm, and phenyl-H signals in the spectrum moved by less, only 0.12 ppm. The signal of the methyl group in the acetyl residue appeared at 1.94 ppm (Fig. 2). In the HMBC experiment, long range coupling was observed from 3-H of APP to carbonyl of the acetyl group. These results indicated that the purified compound was *N*-acetyl-APP.

Production of Ac-APP by Cell-free Extracts

To investigate whether the Ac-APP produced by growing cells was produced by an enzyme, cell-free extacts were prepared by ammonium sulfate fractionation as described in Materials and Methods. When the enzyme reactions were carried out using DL-APP and acetyl coenzyme A as a substrate and the reaction products were analyzed by HPLC with the retention time, the enzyme activity appeared in the 50~80% $(NH_4)_2SO_4$ saturation

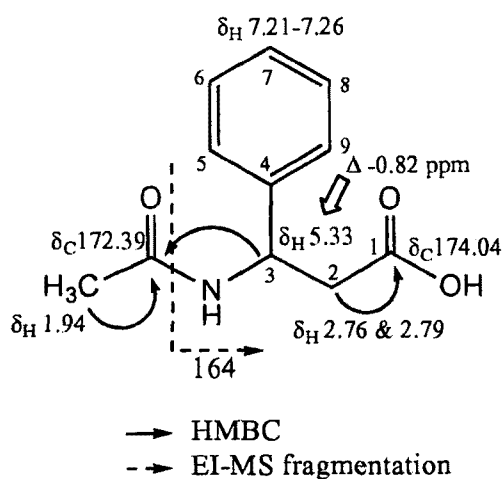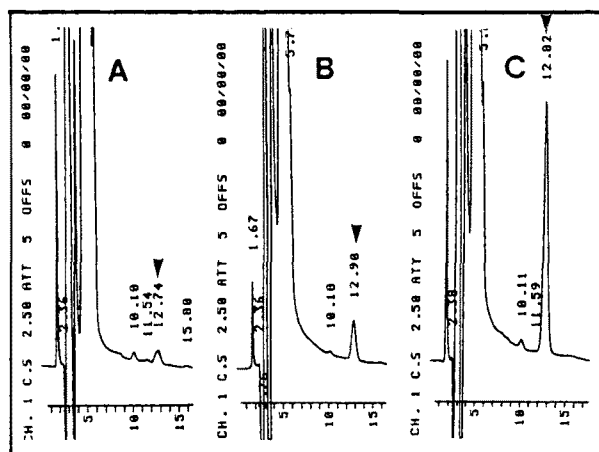Fig. 2. Identification of Ac-APP by NMR data and EI-MS fragmentation.

Fig. 3. *N*-Acetyltransferase activities of $(NH_4)_2SO_4$ saturation fractions analyzed by HPLC.

A, control (no addition of Ac-CoA); B, 0~50% saturation; C, 50~80% saturation. Arrow indicates the Ac-APP formed by enzyme reaction.

fraction (Fig. 3).

The cell-free reaction is dependent on APP, acetyl-CoA and active enzyme extract. Table 1 shows that the omission of either APP or acetyl-CoA prevents Ac-APP formation. Furthermore, boiling of the extract eliminates the conversion. These results indicate that APP is acetylated by transferring the acetyl group from acetyl-CoA to the amino residue of APP via the cell-free extract. Formation of Ac-APP by the cell-free extract was linear for the 4 h duration of the experiment (Fig. 4). As shown in Fig. 4, the reaction rates of the acetyltransferase for substrate APP was higher than that for L-phenylalanine, suggesting that the acetyltrasferase has a specificity for substrates.

Enantioselective *N*-Acetylation of APP by the Cell-free Extracts

The Ac-APP produced by enzyme reaction of cell-free extracts was of a D-configuration: $[\alpha]_D+47.5°$ (MeOH) at 20°C. According to the literature (Dictionary of Organic Compounds, Chapman & Hall, 1982), $[\alpha]_D$ values of *N*-methyl-L-APP and *N*-formyl-D-APP were -47° and +116.4°, respectively (Table 2). The $[\alpha]_D$ value of *N*-Ac-D-APP was not available elsewhere; the compound was hydrolyzed with 6 N HCl to obtain APP. The APP obtained

Table 1. Dependence of Ac-APP formation on acetyl-CoA, APP, and cell-free extract.

Enzyme source	Acetyl CoA	APP	Ac-APP formed (nmol/ml per min)
Cell-free extract	+	+	199.3
Cell-free extract	−	+	10.4
Cell-free extract	+	−	< 10
Boiled cell-free extract	+	+	< 10

Ac-APP formation by enzyme reaction is represented as nmole of Ac-APP formed per min in 1 ml of reaction mixture. +, added; −, not added.
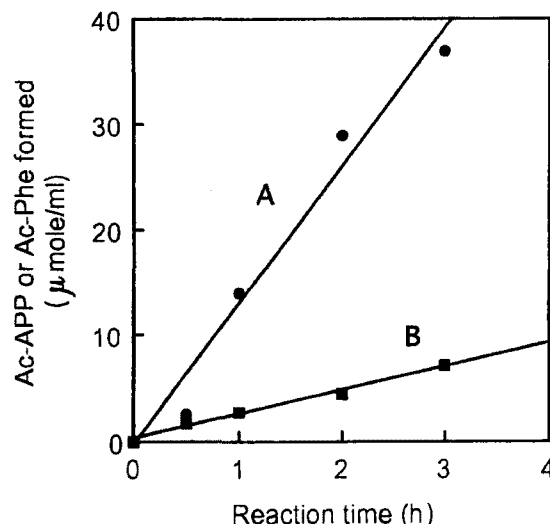

Fig. 4. Dependence of Ac-APP (A) and Ac-Phe (B) formation on incubation time.

Table 2. Comparison of $[\alpha]_D$ values of APP and *N*-substituted APP.

Compound	Source	c	$[\alpha]_D^{20}$	Configuration
N-Ac-APP	This study	0.51 (MeOH)	+47.5	D
N-Ac-APP	Chemical synthesis	0.28 (MeOH)	0	DL
APP	−	− (H₂O)	+7.0	D
APP	−	− (H₂O)	− 7.5	L
N-Me-APP	−	1.70 (MeOH)	− 47.0	L
N-Formyl APP	−	− (EtOH)	+116.4	D

Referred to Dictionary of Organic Compounds, vol. 1. 1982. Chapmann and Hall.

by acid hydrolysis was also D-configuration: $[\alpha]_D+6.5°$ (H_2O) at 20°C, lit. +7° (H_2O), optical purity 92% enantiomeric excesses (**ee**). These results indicate that the acetyltransferase in the cell extract is stereospecific for D-APP. The stereospecificity of the acetyltransferase for substrate (D-APP) could be used for the optical resolution of a DL-APP enantiomeric mixture. Work is in progress to study the purification and characterization of the enzyme and to investigate the physiological function of Ac-APP or acetyltransferase in the cultural environment of the bacterium and its potential applications.

Acknowledgement

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