Biosynthesis of Biotin-vitimers from Pelargonic Acid by *Pseudomonas* sp. Strain 393

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The biosynthesis of biotin-vitimers from pelargonic acid by *Pseudomonas* sp. strain 393 was investigated. The main product of biotin-vitimers from pelargonic acid was desthiobiotin. The addition of streptomycin or L-alanine enhanced accumulation of desthiobiotin in culture fluid. Propionic, pimelic and azelaic acids were identified as main metabolites from pelargonic acid. When propionic acid was incubated with resting cells, pelargonic and azelaic acids were formed. The biosynthetic pathway of pelargonic acid to pimelic acid was also studied.

In the previous paper,¹⁾ we reported the direct participation of pelargonic acid (PeA) in biotin biosynthesis; the radioactivity of PeA- $(1-^{14}\text{C})$ was incorporated into biotin-vitimers such as 7-keto-8-aminopelargonic acid (KAPA) and desthiobiotin (DTB) by *Pseudomonas* sp. strain 393. After that, we showed formation of pimelic acid (PmA), which is known as an important precursor of biotin, from PeA in resting cells of this organism.²⁾ Although the biotin biosynthesis from PmA to DTB by microorganisms has been well investigated, the biosynthetic pathway to PmA is still unsettled. This paper deals with the cultivation conditions for formation of biotin-vitimers from PeA and the role of PeA as a precursor of PmA in *Pseudomonas* sp. strain 393.

MATERIALS AND METHODS

Microorganism. *Pseudomonas* sp. strain 393, which utilized PeA as a sole carbon source, was used throughout this work.

Medium. The medium was composed of 1% PeA, 0.3% $(\text{NH}_4)_2\text{SO}_4$, 0.2% K_2HPO_4 , 0.1% KH_2PO_4 and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in tap water. The initial pH was adjusted to 7.0.

Cultivation. To determine the ability of biotin-vitamer formation, 4 ml of the medium in a test tube (16 mm) was incubated at 28°C for one to three days under shaking (230 rpm). The content of biotin-vitimers in culture broth was determined. To prepare resting cells the organism was preincubated in 4 ml of the medium, and then inoculated into 400 ml of the medium in a 2-liter shaking flask. The flask was incubated at 28°C for two to four days with shaking (130 rpm).

Growth measurement. The growth of the organism was routinely estimated by measuring the turbidity with a Hiranuma Photometer EPO-B type at 470 nm.

Preparation of resting cells and cell-free extracts. *Pseudomonas* sp. strain 393 was grown in a 2-liter flask containing 400 ml of the medium on a shaker at 28°C for two days. The cells were harvested by centrifugation, and washed twice with 0.85% saline solution. The washed cells were used as resting cells. To prepare cell-free extracts the washed cells were suspended in M/200 potassium phosphate buffer (pH 7.0) containing 1.2 mM 2-mercaptoethanol and subjected to the action of a Kaijodenki ultrasonic oscillator (19 kHz) for 10 min below 10°C. The undestroyed cells and debris were removed by centrifugation at $10,000 \times g$ for 20 min. The supernatant solution thus obtained was used as the cell-free extract. Protein concentration was determined by the method of Lowry *et al.*³⁾

Determination and identification of biotin-vitimers. Biotin-vitimers were quantitatively determined by an assay with *Saccharomyces cerevisiae*.⁴⁾ Biotin-vitimers syn-

thesized were identified by paper chromatography.⁵⁾

Separation and identification of products from PeA by gas-liquid chromatography. *Pseudomonas* sp. strain 393 was grown at 28°C for 45 hr in 400 ml of the medium in a 2-liter flask. After growth, the cultures were centrifuged to remove cells. The supernatant obtained was concentrated to a small volume *in vacuo* and organic acids were extracted twice with 30 ml of ether. The extract was dehydrated with anhydrous Na₂SO₄, evaporated to about one-tenth volume, methylated with BF₃-methanol reagent,⁶⁾ and analyzed by gas-liquid chromatography. The conditions for gas chromatography were: Instrument, Shimadzu GC-4BP (FID); a glass column (3 mm i.d. × 2.0 m) packed with 10% DEGS on Shimalite W (60~80 mesh); column temp., initial 100°C, final 200°C; programming rate, 5°C/min; injection and detector temp., 240°C; carrier gas, nitrogen at 60 ml/min; hydrogen, 50 ml/min; air 800 ml/min; and sensitivity × range, 10² × 64 (1.6 × 10⁻⁹ ampere in full scale).

Enzyme activities.

(1) *ω*-Oxidation of PeA. The reaction mixture contained 0.23 μmol of NADH or NAD, 0.23 μmol of FeSO₄·7H₂O, 76 mmol of Tris-HCl buffer (pH 7.6), 3.8 μmol of PeA and cell-free extracts (700 μg of protein) in a final volume of 1.0 ml.⁷⁾ Enzyme activity was measured by following the rate of NADH oxidation with a Hitachi Model 124 Type Spectrophotometer at 340 nm at 25°C.

(2) *KAPA synthetase*. The incubation mixture contained 1 μmol of PmA, 10 nmol of CoA, 1 μmol of ATP, 1 μmol of MgCl₂, 5 μmol of L-alanine, 10 nmol of pyridoxal-P, 10 μmol of potassium phosphate buffer (pH 7.0) and cell-free extracts (200 μg of protein) in a final volume of 120 μl. It was incubated at 37°C for 3 hr.⁸⁾ KAPA was determined by an assay with *Saccharomyces cerevisiae*.⁴⁾

(3) *Acyl-CoA synthetase*. The assay mixture contained 200 μmol of fatty acid, 20 μmol of CoA, 250 μmol of ATP, 2 mmol of MgCl₂, 100 mmol of Tris-HCl buffer (pH 7.6) and cell-free extracts (750 μg of protein) in a final volume of 500 μl. Incubation was performed at 35°C. Enzyme activity was spectrophotometrically measured by the following assays, (I), (II) and (III): assay (I) measured the rate of CoA consumption from the decrease in absorbance at 412 nm resulting from the reaction of CoA with DTNB⁹⁾; assay (II) measured the rate of thiolester formation from the increase in absorbance at 540 nm resulting from the reaction of thiolester with hydroxylamine¹⁰⁾; assay (III) measured the rate of thiolester formation from the increase in absorbance at 232 nm depending on thiolester itself.¹¹⁾

Chemicals. KAPA was a kind gift from Drs. H. Yamada, Y. Tani and Y. Izumi, Kyoto University. Other chemicals used in this work were commercial products.

RESULTS

Accumulation of biotin-vitamer from PeA

As preliminarily reported,¹⁾ *Pseudomonas* sp. strain 393 which utilized PeA as a sole carbon source formed DTB as a main biotin-vitamer, together with another product of KAPA in culture broth. Moreover, the effect of various fatty acids on accumulation of biotin-vitamer showed that PeA was the most effective of them, while PmA and azelaic acid (AeA) were not effective. The time course of biotin-vitamer accumulation from PeA was examined. We found that biotin-vitamer derived from PeA were accumulated gradually along with growth of the organism (Fig. 1). After 60 hr cultivation, biotin-vitamer accumulation decreased. On the other hand, biotin-vitamer accumulation from PeA in the presence of propionic acid (PoA) was suppressed during 60 hr cultivation. After that, biotin-vitamer accumulation increased gradually and reached the same maximum level as the accumulation from PeA. Now, the growth of *Pseudomonas* sp. strain 393 on PeA (1%) medium was almost the same as the growth on PeA (1%) medium in the presence of PoA (0.1%).

FIG. 1. Time Courses of the Accumulation of Biotin-vitamer from PeA and PoA.

---○---, growth on PeA (1%) medium; —●—, biotin-vitamer accumulation on PeA (1%) medium; —●—, biotin-vitamer accumulation on PeA (1%) medium in the presence of PoA (0.1%).

TABLE I. EFFECT OF AMINO ACIDS ON THE ACCUMULATION OF BIOTIN-VITAMERS FROM PeA

Cultivation was performed at 28°C for 64 hr on a shaker (230 rpm).

Amino acids added (0.1%)	Growth (Turbidity at 470 nm)	Biotin-vitamers formed ($\mu\text{g/ml}$)
None	0.81	8.0
L-Serine	0.75	23.0
L-Alanine	0.87	36.0
L-Aspartic acid	0.76	14.5
L-Glutamic acid	0.89	18.0
L-Lysine	0.73	15.0
L-Ornithine	0.78	23.0
L-Proline	0.81	23.0
L-Leucine	0.77	23.0
L-Valine	0.79	23.0
L-Cystine	0.48	15.0
L-Methionine	0.85	15.0
L-Phenylalanine	0.82	14.5

Effects of nitrogen sources and amino acids on accumulation of biotin-vitamers

The influence of nitrogen sources on biotin-vitamer accumulation from PeA was investigated. Urea served as a good nitrogen source (biotin-vitamers formed, 20 $\mu\text{g/ml}$). The effects of casamino acids and peptone on biotin-vitamer accumulation were nearly equal (10 $\mu\text{g/ml}$) to those of ammonium sulfate and ammonium chloride. In the following experiment, the effect of various amino acids on biotin-vitamer accumulation was examined. Several amino acids such as L-valine, L-alanine, L-proline and L-ornithine stimulated biotin-vitamer formation (Table I). The participation of L-alanine in the formation of the ureido ring of the DTB molecule was suggested first by Iwahara *et al.*¹¹⁾ with the resting cell system of *Bacillus sphaericus*, and finally demonstrated by Izumi *et al.*^{12,13)} who discovered the enzyme, KAPA synthetase, which catalyzes the synthesis of KAPA from pimel-CoA and L-alanine.

Effect of antibiotics on the accumulation of biotin-vitamers from PeA

The effect of 5 kinds of antibiotics on biotin-

vitamer accumulation was investigated. Neither penicillin G, tetracycline nor kanamycin (100 $\mu\text{g/ml}$ each was added to medium) was effective. Chloramphenicol and streptomycin enhanced biotin-vitamer accumulation. When streptomycin (10 $\mu\text{g/ml}$) was added to the medium at the initial stage of growth, biotin-vitamer accumulation throughout the cultivation increased about two-fold. The biotin-vitamers accumulated in the presence of streptomycin were separated by paper chromatography, and detected with *Saccharomyces cerevisiae*. DTB and KAPA were identified as main biotin-vitamers.

Identification of metabolites derived from PeA during cultivation

In the previous study,¹⁾ we found that *Pseudomonas* sp. strain 393 synthesizes mainly DTB and KAPA as biotin-vitamers from PeA. To clarify the biosynthetic pathway from PeA to KAPA, metabolites from PeA accumulated in culture fluid were checked by gas-liquid chromatography. Monocarboxylic acids of 8, 7 and 3 carbon chain lengths and dicarboxylic acids of 9, 7, 6, 5, 4 and 3 carbon chain lengths were formed from PeA during 45 hr cultivation. Among degradation products, PoA and AeA were mainly accumulated. Moreover, PmA, which is an important precursor of biotin, was also found. This suggests that the formation of PmA from PeA is due to the presence in cells of two oxidation mechanisms: One is AeA formation by ω -oxidation of PeA, and the other is the PmA formation by β -oxidation of AeA. On the other hand, PoA is probably formed as an end product of PeA through its β -oxidation.

Biosynthesis of biotin-vitamers from PeA by resting cells

The biosynthesis of biotin-vitamers from PeA by resting cells also was investigated. The effects of cell concentration and incubation time on biotin-vitamer biosynthesis were examined. A linear relationship was found between the *Saccharomyces cerevisiae* activity and the amount of resting cells ranging from 5

to 60 mg, with a reaction mixture incubated for less than 6 hr. However, even if PeA was absent from the reaction mixture, biotin-vitamins were slightly synthesized at a high concentration of cells. In the previous study,²⁾ we showed the formation of PmA and AeA from PeA in resting cells of this organism by gas-liquid chromatography. When PoA is incubated with resting cells, PeA and AeA are formed in the reaction mixture during 3 hr incubation.

Enzyme activities related to the biosynthetic pathway from PeA to KAPA

(1) *ω -Oxidation of PeA.* Several experiments on the decomposition of PeA were done by incubating PeA with cofactors and cell-free extracts of *Pseudomonas* sp. strain 393 until AeA was formed. AeA was checked by gas-liquid chromatography. Among NAD, NADH, NADP and NADPH, only NADH caused disappearance of PeA. Figure 2 shows that with PeA as a substrate cell-free extracts catalyze consumption of NADH with incubation time at 25°C. The fate of the PeA moiety during incubation in the presence of NADH of cell-free extracts was determined by gas-liquid chromatography. During 90 min incubation AeA and PmA were formed. *ω -Oxidation of heptanoic acid (C_7)* under the described conditions was examined, but no significant formation of PmA from C_7 was observed.

(2) *KAPA synthesis.* To study biotin-vitamer biosynthesis from PmA, we examined KAPA synthesis from PmA and L-alanine. KAPA was synthesized in the complete system consisting of PmA, CoA, ATP, $MgCl_2$, L-alanine and pyridoxal-P. Without cell-free extracts KAPA was not entirely formed.

(3) *Fatty acid activating enzyme.* The activity of CoA thiolester formation of PoA by cell-free extracts was examined by assays (I) and (II) described in the text. Figure 3 shows that during the rapid initial part of the reaction an almost linear relationship was found between CoA thiolester formation (or CoA disappearance) and incubation time. Subsequent

FIG. 2. NADH Consumption during Decomposition of PeA by Cell-free Extracts.

—○—, without addition of PeA; —●—, with addition of PeA.

FIG. 3. CoA Thiolester Formation and CoA Disappearance during Incubation of Cell-free Extracts with PoA and CoA.

—●—, formation of CoA thiolester determined by assay (II) described under MATERIALS AND METHODS; —○—, CoA disappearance determined by assay (I) described under MATERIALS AND METHODS.

experiments on the CoA thiolester formation were done with mono- and dicarboxylic acids as substrates. The activity was highest for the C_3 chain length (PoA) and decreased in the order $C_3 > C_9 > C_5 > C_6 > C_4 > C_5$ (glutaric acid) $> C_4$ (succinic acid) $> C_8 > C_9$ (AeA).

DISCUSSION

Since earlier studies showed that PmA is an intermediate in the oxidative degradation of

FIG. 4. PeA Metabolism in Biotin Biosynthesis by *Pseudomonas* sp. Strain 393.

AeA by *Micrococcus* sp. strain A133,¹⁴⁾ we have investigated the participation of several compounds in biotin biosynthesis. We have reported that oleic acid,^{15,16)} salicylic acid,¹⁷⁾ PeA,¹⁾ α -picolinic acid¹⁸⁾ and linoleic acid¹⁹⁾ could serve as precursors of biotin in some bacteria and yeasts, and that AeA and PmA were identified as products derived from oleic acid,²⁰⁾ PeA²⁾ and α -picolinic acid.¹⁸⁾

Pseudomonas sp. strain 393 could utilize not only PeA but also PoA as a sole carbon source, and formed DTB as a biotin-vitamer in culture fluid from those acids. The metabolites of PeA on 45 hr cultivation of this organism were PoA and AeA. The yields of PoA and AeA varied widely depending on the cultivation time. At 20 to 30 hr cultivation PoA was the main product, whereas after 40 hr cultivation AeA, PmA and malonic acid were formed. When acrylic acid (0.1 mg/ml of medium added), which is reported to be an inhibitor of β -oxidation,²¹⁾ was added to the medium at the initial stage of growth, this organism utilized PeA only after an about 20 hr delay. Moreover, with addition of acrylic acid at 28 hr cultivation biotin-vitamer accumulation had decreased by over 50% at 45 hr cultivation, but after then increased gradually. This fact suggests that β - and ω -oxidations of PeA may occur in *Pseudomonas* sp. strain 393. In addition, AeA formation from PeA was observed in the reaction mixture after incubation with resting cells in the presence of NADH as reported previously.²⁾

The above evidence leads to the following hypothesis for the role of PeA as a precursor of PmA in *Pseudomonas* sp. strain 393 (Fig. 4). The main system for energy supply is a β -oxidation which results in PoA formation. On the other hand, the biosynthetic pathway to biotin-vitimers is due to an ω -oxidation, followed by a β -oxidation to give PmA. When PoA is used as an energy source, the biosynthetic system of a fatty acid occurs to form PeA. Table I shows the marked effect of L-alanine on biotin-vitamer accumulation. Since KAPA is synthesized from PmA, CoA and L-alanine, the biosynthetic pathway to DTB from PeA must be *via* PmA as reviewed.^{22~24)}

Ogata *et al.*²⁵⁾ have reported the action of an antibiotic, acidomycin, which markedly enhances biotin-vitamer accumulation in various microorganisms. However, we have no information on the regulation of biotin-vitamer accumulation by streptomycin.

REFERENCES

- 1) M. Ohsugi and A. Baba, *Agric. Biol. Chem.*, **39**, 731 (1975).
- 2) M. Ohsugi, Y. Inoue, M. Ukisu, N. Imai and H. Deguchi, *Agric. Biol. Chem.*, **46**, 1391 (1982).
- 3) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 4) E. E. Snell, R. E. Eakin and R. J. Williams, *J. Am. Chem. Soc.*, **62**, 175 (1940).
- 5) K. Ogata, T. Tochikura, S. Iwahara, K. Ikushima, S. Takasawa, A. Nishimura and M. Kikuchi, *Agric. Biol. Chem.*, **29**, 895 (1965).
- 6) W. R. Morrison and L. M. Smith, *J. Lipid Res.*, **5**,

- 600 (1964).
- 7) M. Kusunose, E. Kusunose and M. J. Coon, *J. Biol. Chem.*, **239**, 1374 (1964).
 - 8) Y. Izumi, H. Morita, Y. Tani and K. Ogata, *Agric. Biol. Chem.*, **37**, 1327 (1973).
 - 9) P. A. Srere, "Methods in Enzymology," ed. by J. M. Lowenstein, Vol. 13, Academic Press, New York, 1969, p. 3.
 - 10) F. Lipmann and L. C. Tuttle, *Biochim. Biophys. Acta*, **4**, 301 (1950).
 - 11) S. Iwahara, T. Tochikura and K. Ogata, *Agric. Biol. Chem.*, **30**, 1076 (1966).
 - 12) Y. Izumi, H. Morita, Y. Tani and K. Ogata, *Agric. Biol. Chem.*, **36**, 510 (1972); *Agric. Biol. Chem.*, **37**, 1327 (1973).
 - 13) Y. Izumi, K. Sato, Y. Tani and K. Ogata, *Agric. Biol. Chem.*, **37**, 1335 (1973).
 - 14) K. Ogata, T. Tochikura, M. Ohsugi and S. Iwahara, *Agric. Biol. Chem.*, **30**, 176 (1966).
 - 15) M. Ohsugi, H. C. Yang and K. Ogata, *Agric. Biol. Chem.*, **36**, 1285 (1972).
 - 16) M. Ohsugi and Y. Ishikawa, *Agric. Biol. Chem.*, **39**, 559 (1975).
 - 17) M. Ohsugi, M. Nakazawa and K. Ogata, *Agric. Biol. Chem.*, **36**, 1293 (1972).
 - 18) M. Ohsugi, Y. Inoue, K. Takami and M. Namikawa, *Agric. Biol. Chem.*, **45**, 1879 (1981).
 - 19) Y. Inoue, N. Iwatani and M. Ohsugi, *Vitamins (Japan)*, **56**, 241 (1982).
 - 20) M. Ohsugi and Y. Inoue, *Agric. Biol. Chem.*, **45**, 2355 (1981).
 - 21) G. J. E. Thijsse, *Biochim. Biophys. Acta*, **84**, 195 (1964).
 - 22) M. A. Eisenberg, "Advances in Enzymology," Vol. 38, Interscience, New York, 1973, p. 317.
 - 23) Y. Izumi and K. Ogata, "Advances in Applied Microbiology," Vol. 22, Academic Press, New York, 1977, p. 145.
 - 24) Y. Izumi, Y. Tani and H. Yamada, *Bull. Inst. Chem. Res., Kyoto Univ.*, **58**, 433 (1980).
 - 25) K. Ogata, Y. Izumi and Y. Tani, *Agric. Biol. Chem.*, **37**, 1079 (1973).
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