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201. Yukio Kameda and Katsuhiko Matsui*1 : Studies on Acylase Activity and Microorganisms. XXII.*2 Isolation of Soil Bacteria Capable of Producing δ-Ornithine Acylase (5-N-Acyl-ornithine Amidohydrolase).*3

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Screening experiments for δ-ornithine acylase activity in soil bacteria have been carried out for the purpose of finding out new enzymes. A number of bacteria were cultured on the medium containing 5-N-acyl-L-ornithine, and the cultural broth were examined the liberated ornithine by paper chromatography. Then, δ-ornithine acylase activities of these bacteria, which appeared a distinct spot of ornithine, were measured by estimation of liberated ornithine after incubation of 5-N-acyl-L-orthinine with their acetone powder. As a result, the δ-ornithine acylase activity was observed in some soil bacteria and a strain (KT 801) showed the highest activity. KT 801 was isolated by using the synthetic medium containing 5-N-benzoyl-L-ornithine as a sole source of carbon and confirmed to belong in *Pseudomonas* group.

δ-Ornithine acylase of KT 801 hydrolyzes 6-N-acyl-L-lysine and 5-N-acyl-L-ornithine, although KT 83 (*Pseudomonas* sp.) hydrolyzes 6-N-acyl-L-lysine but not 5-N-acyl-L-ornithine, and KT 511 (*Brevibacterivm* sp.) hydrolyzes ω-phenylacetyl derivatives of lysine and ornithine but not the benzoyl derivatives.

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Although a number of reports[1] have been published on the distribution and enzymatic properties of α-amino acylase which can hydrolyze α-N-acyl amino acids, little is known about ω-amino acylase which can hydrolyze ω-N-acyl derivatives of α,ω-diamino acids. Paik, *et al.*[2] reported for the first time on ε-lysine acylase, which hydrolyzes 6-N-acyllysine, in rat tissues. The authors[3] found independently the hydrolytic activity toward 6-N-benzoyl-L-lysine in a bacterium (KT 83) of *Pseudomonas* species isolated from soil, and this ε-lysine acylase was purified and its properties were studied by Wada.[4] Chibata, *et al.*[5,6] also studied ε-lysine acylase activities in *Aspergillus oryzae* and *Achromobacter pestifer*. But an enzyme which hydrolyzes ω-acyl derivatives of ornithine, 2,4-diaminobutyric acid, and 2,3-diaminopropionic acid, has not yet been reported.

During the course of investigation in this laboratory on α-amino acylase of soil bacteria, the authors observed that there are many α-amino acylases which have different substrate specificities.[3,7~9] These results suggest that a new amino acylase may be found out from soil bacteria by selection of suitable condition. Then, the authors investigated the occurrence of δ-ornithine acylase (5-N-acylornithine aminohydrolase) activity in

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soil bacteria for the purpose of finding out new enzymes. The screening experiments in organisms isolated from soil and in preserved bacteria in our laboratory, have been carried out by paper chromatographic method as employed in screening of ε-lysine acylase by Chibata, *et al.*[5] The strains which appeared a distinct spot of ornithine were grown in bouillon, prepared to acetone powder, and δ-ornithine acylase activities were measured by estimation of liberated ornithine after incubation of 5-N-acylornithine with the acetone powder at 37°. As a result of present experiments, a strain showing extremely high δ-ornithine acylase activity was isolated and this strain was identified to belong in *Pseudomonas* group.

Materials and Methods

Compounds used

5-N-Benzoyl-L-ornithine——5-N-Benzoyl-L-ornithine was prepared by the method of McGilvery[10] which consists in treating the copper complex of L-ornithine with benzoyl chloride, and the copper was removed by hydrogen sulfide.

5-N-Phenylacetyl-L-ornithine——5-N-Phenylacetyl-L-ornithine was prepared in substantially the same manner as 5-N-benzoyl-L-ornithine. Excess basic $CuCO_3$ (6 g.) was added gradually to a boiling solution of L-ornithine·HCl (5 g.). After cooling, the solution was filtered, 2*N* NaOH (15 ml.) was added, and the dark blue solution cooled in ice. Phenylacetylchloride (5 g.) and 2*N* NaOH (15 ml.) were added in portions over 30 min. with shaking and cooling. The Cu complex, which separased as a blue precipitate, was filtered off and washed well with H_2O and EtOH. It was suspended in 1 L. of H_2O, and H_2S passed in with mechanical shaking. The solution was then brought to the boil and filtered hot. The filtrate was concentrated *in vacuo*, and resulting precipitate was filtered off. Recrystallization from H_2O afforded 2.5 g. (34%) of 5-N-phenylacetyl-L-ornithine, m.p. 266° (decomp.), $[\alpha]_D$: +22°(C=2, 5*N* HCl). *Anal.* Calcd. for $C_{13}H_{18}O_3N_2$: C, 62.38; H, 7.25; N, 11.19. Found : C, 62.09; H, 7.35; N, 11.22.

6-N-Benzoyl-L-lysine——6-N-Benzoyl-L-lysine was prepared by the method of Kurtz.[11]

6-N-Phenylacetyl-L-lysine——6-N-Phenylacetyl-L-lysine was prepared in the same manner as above described 5-N-phenylacetyl-L-ornithine. Recrystallization from H_2O afforded 1.2 g. (38%) of 6-N-phenylacetyl-L-lysine, m.p. 263° (decomp.), $[\alpha]_D$: +14°(C=2, 5*N* HCl) from 2.2 g. of L-lysine·HCl. *Anal.* Calcd. for $C_{14}H_{20}O_3N_2$: C, 63.61; H, 7.63; N, 10.60. Found : C, 63.41; H, 7.76; N, 10.83.

Isolation of Soil Bacteria Capable of Metabolizing 5-N-Benzoyl-L-ornithine or 5-N-Phenylacetyl-L-ornithine——The constituents of the culture medium for the isolation of soil bacteria are as follows : Carbon source (5-N-benzoyl-L-ornithine or 5-N-phenylacetyl-L-ornithine), 0.2 g.; NH_4Cl, 0.1 g.; K_2HPO_4, 0.1 g.; $MgSO_4 \cdot 7H_2O$, 0.05 g.; 1% $CaCl_2$, 2 drops; 1% $FeCl_3$, 1 drop; H_2O, 100 ml.; (pH 7.2).

0.2~0.5 g. of a soil sample, taken from the herbal garden of this University or other parts, was inoculated into 10 ml. of the above culture medium and incubated at 27° for 2~6 days. If luxuriant growth of bateria occurred, a loop of the culture fluid was transferred to a new culture medium having the same constituents. Such transplantation was repeated at least twice. The bacterial suspension of the last generation was then planted in an agar medium, containing 1.5% agar in the above culture medium. Culture experiments were carried out in order to determine whether the microbe isolated from the agar plate could grow in the above medium.

Screening Method of the Enzyme Activity——The culture media for the isolation of soil bacteria were distributed in amounts of 10 ml. to test tubes and sterilized. After inoculation with a loopful of the organism, incubation was carried out at 27°. Routine identification and an estimation of liberated amino acid was made by paper chromatography of the cultural broth after 2 and 4 days. Paper chromatography was carried out with the ascending method using Tōyō filter paper No. 51 and BuOH:AcOH:H_2O=4:1:2 as the solvent. After development for about 15hr., the paper chromatogram was treated with a 0.3% ninhydrin EtOH solution. Estimation of δ-ornithine acylase activity was made by detection of the spot of ornithine.

Assay of the Enzyme Activity——Enzyme reaction was carried out by incubating a mixture of 1 ml. of 0.01 *M* substrate (5-N-Bz-L-Orn. or 5-N-Pa-L-Orn.), 1 ml. of 0.1 *M* phosphate buffer (pH 7.0), and 10 mg. of acetone powder at 37°. The liberated ornithine was measured by Chinard's acidic colorimetric ninhydrin method[12] with a minor modification.[13,4] After 2 and 4 hr., 0.4 ml. of the incubated solution was added to 1.6 ml. of AcOH and 1.0 ml. of a 2% solution of ninhydrin in AcOH, and the mixture was immersed in boil-

10) R.W. McGilvery, P.P. Cohen : J. Biol. Chem., **183**, 179 (1950).
11) A.C. Kurtz : *Ibid.*, **180**, 1253 (1949).
12) F.P. Chinard : *Ibid.*, **199**, 91 (1952).
13) I. Chibata, T. Ishikawa, T. Tosa : Bull. Agr. Chem. Soc. Japan, **24**, 37 (1960).

ing water for 30 min. The solution was diluted with 7.0 ml. of 50% EtOH, and the absorption intensity after 5 fold dilution with 50% EtOH was measured at 515 mμ, giving a measure of the free ornithine produced by the enzymatic hydrolysis. Enzyme activity was expressed in terms of micromoles of liberated ornithine per hr. per 10 mg. acetone powder of soil bacteria.

Preparation of Acetone Powder of Soil Bacteria——Soil bacteria were grown in 200 ml. of bouillon (pH 7.2) with reciprocal shaking (108 c/min., stroke 7 cm.), at 27° for 2 days. The cells were then harvested by centrifugation and washed with dist. H_2O. The cells were poured into 200 ml. of acetone cooled to −5°, and the mixture was stirred for 5 min. The solid were collected by suction, washed with cold acetone and then ether, and dried in a desiccator.

Results

δ-Ornithine Acylase Activity in Soil Bacteria

The bacteria which have been isolated from soil and preserved in our laboratory, were tested for δ-ornithine acylase by screening experiments. Appearance of a distinct spot of ornithine indicating the occurrence of δ-ornithine acylase was observed in about 9 kinds of soil bacteria. Then, the strains which appeared a distinct spot of ornithine were grown in bouillon. The cells were harvested by centrifugation, prepared to the acetone powder, and measured the δ-ornithine acylase activities. As shown in Table I, most of the soil bacteria, which exhibited an ornithine spot in screening experiments, showed δ-ornithine acylase activity also in acetone powder. A strain (KT 801) was found to have the highest δ-ornithine acylase activity among the tested bacteria.

Table I. δ-Ornithine Acylase Activities of Soil Bacteria

Strain	δ-Ornithine acylase activities[a]		Strain	δ-Ornithine acylase activities[a]	
	5-N-Benzoyl-L-ornithine	5-N-Phenylacetyl-L-ornithine		5-N-Benzoyl-L-ornithine	5-N-Phenylacetyl-L-ornithine
KT 511	0	3.4	KT 804	0.8	0.2
KT 515	0	3.0	KT 805	0	1.0
KT 801	50.4	5.0	KT 806	0	0.5
KT 802	3.5	1.0	KT 807	0.5	1.0
KT 803	0.3	0			

a) Enzyme activities are expressed in terms of μ*M* of liberated ornithine per hr. per 10 mg. of acetone powder.
Digest consisting 1 ml. of 0.01*M* substrate, 1 ml. of 0.1*M* phosphate buffer (pH 7.0), and 10 mg. of the acetone powder is incubated at 37°. Activities are measured by the procedure described in "Method."

Identification of the Strain (KT 801) producing the Most Potent δ-Ornithine Acylase

KT 801, which showed the highest δ-ornithine acylase activity in the above experiments, was obtained from a soil of herbal garden of this University by using of medium containing 5-N-benzoyl-L-ornithine as a sole source of carbon. It was Gram negative straight rods, 0.8~1.0×1.2~1.5 μ, motile by single polar flagella (Electron microscopy photograph, negative staining by phosphotungstic acid). KT 801 grew well 5-N-benzoyl-L-ornithine, L-ornithine, L-glutamate, L-aspartate, L-alanine, benzoate, *p*-hydroxybenzoate, phenylacetate, acetate, succinate, fumarate, and lactate etc. as a sole source of carbon. But no growth occcurred with glucose, lactose, sucrose, glycerine, citrate, salicylate, glycine, L-leucine, L-threonine, L-methionine, L-lysine, L-histidine, L-proline, and L-phenylalanine as a sole source of carbon at 27° for 6 days. Gelatin medium was not liquefied when KT 801 was incubated at 22° for 6 days, and nitrite was not produc-

ed from nitrate when it was grown in peptone nitrate medium at 27° for 5 days. Indole was not produced when it was grown in peptone medium at 27° for 2 days. These taxonomic nature of KT 801 were summarized in Table II. According to the Bergey's Manual of Determinative Bacteriology, this strain was confirmed to belong in *Pseudomonas* group.

Table II. Taxonomic Nature of the Bacterium (KT 801) producing δ-Ornithine Acylase

Size : 0.8~1.0×1.2~1.5 μ	Nitrites : Not produced from nitrates.	
Shape : Rods.	Methyl red test : Negative.	
Gram : Negative.	Indole : Not produced.	
Flagella : Polar flagella.	Glucose ammonium solution	Not utilized
Motility : Slow, undulatory motion, occurring in pairs.	Glucose nitrate solution	Not utilized
	Succinate nitrate solution	Not utilized
Gelatin : No liqueified.	Glutamate solution	Utilized
Litmus milk : Medium becomes slimy alkaline.	*p*-Hydroxybenzoate solution	Utilized

Discussion

In connection with ω-amino acylase which hydrolyzes ω-acyl derivatives of α,ω-diamino acids, there are some reports on ε-lysine acylase, but an enzyme which hydrolyzes δ-acyl derivatives of ornithine has not yet been reported. Then, the authors carried out the investigation on the distribution of δ-ornithine acylase activity in soil bacteria. For this purpose, the respective soil bacteria were cultured on the medium containing 5-N-benzoyl- or 5-N-phenylacetyl-L-ornithine, and screening test of δ-ornithine acylase was carried out by paper chromatographic method. It is also possible that the amino acid fomed by enzymatic hydrolysis are further metabolized by the organisms during incubation. Accordingly, there is a possibility that organisms other than those detected by the present screening procedure may show higher δ-ornithine acylase activity. But it is certain that the present method is simple and useful as a screening procedure to estimate rough tendency of distribution of the enzyme activity. Then, the strains which appeared a distinct spot of ornithine, were measured the δ-ornithine acylase activity with their acetone powder. As a result of present experiment, it was observed that KT 801 has the highest δ-ornithine acylase in tested organisms and KT 511[14] (*Brevibacterium* sp.) which has a penicillin acylase activity, has the hydrolytic activity toward 5-N-phenylacetyl-L-ornithine, but not toward 5-N-benzoyl-L-ornithine. It was reported[4] that KT 83 (*Pseudomonas* sp.) has the hydrolytic activity toward 6-N-benzoyl-L-lysine but

Table III. ε-Lysine Acylase Activities and δ-Ornithine Acylase Activities of Soil Bacteria

Strain	6-N-Bz-L-Lys.	6-N-Pa-L-Lys.	5-N-Bz-L-Orn.	5-N-Pa-L-Orn.
KT 83	9	2.1	0	0
KT 511	0	1.4	0	3.4
KT 801	2.4	0.2	50.4	5.0

The following abbreviations are used in this Table : Bz : Benzoyl, Lys. : Lysine, Pa : Phenylacetyl, Orn. : Ornithine.

Digest consisting 1 ml. of 0.01*M* substrate, 1 ml. of 0.1*M* phosphate buffer (pH 7.0), and 10 mg. of the acetone powder is incubated at 37°. Activities are measured by the procedure described in "Method."

Enzyme activities are expressed in terms of μ*M* of liberated lysine or ornithine per hr. per 10 mg. acetone powder.

14) Y. Kameda, Y. Kimura. E. Toyoura, T. Omori : Nature, **191**, 1122 (1961).

not 5-N-benzoyl-L-ornithine. Then, the authors examined the hydrolytic activities of KT 83, KT 511, and KT 801 toward 6-N-benzoyl-L-lysine, 6-N-phenylacetyl-L-lysine, 5-N-benzoyl-L-ornithine, and 5-N-phenylacetyl-L-ornithine, and the results were revealed in Table Ⅲ. From Table Ⅲ, it is shown that KT 83 hydrolyzes 6-N-acyl derivatives of L-lysine, but not 5-N-acyl-L-ornithine, KT 511 hydrolyzes ω-phenylacetyl derivatives of lysine and ornithine but not the benzoyl derivatives, and KT 801 hydrolyzes 6-N-acyl-L-lysine and 5-N-acyl-L-ornithine. It is suggested that the substrate specificity of ω-amino acylase are different by origin of enzymes.

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