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203. Katsuhiko Matsui*1 : Studies on Acylase Activity and Microorganisms. XXIV.*2 Properties of δ-Ornithine Acylase (5-N-Acylornithine Amidohydrolase).*3

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In order to study the enzymatic properties of the δ-ornithine acylase in KT 801 (*Pseudomonas* sp.), experiments were carried out with the purified enzyme preparation. This enzyme has a pH optimum around 8.2 toward 5-N-benzoyl-L-ornithine. It is inhibited by Co^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Hg^{2+}, EDTA, and *o*-phenanthroline. The inactivation for EDTA is reversed by addition of Ca^{2+} or Mn^{2+} after dialysis. Km value toward 5-N-benzoyl-L-ornithine was calculated to be $1.8\times10^{-4}M$. As a results of the investigation of substrate specificity, it was found that this enzyme hydrolyses not only L form of 5-N-benzoylornithine, but D form in the rate of about one sixth. The ratio of the activities toward L and D form remained constant all through the purification steps. These result strongly suggest that one enzyme has the both activities. Also, susceptible substrate to δ-ornithine acylase require absolutely the presence of a free carboxyl group, the presence of a free α-amino group is desirable for ready susceptibility of the substrate, but it is not an absolute requirement, and the susceptibility are considerably affected by carbon chain length between the carboxyamide group and the free carboxyl group. This enzyme shows no α-amino acylase activity.

On the other hand, δ-ornithine acylase is quantitatively released when EDTA-lysozyme spheroplasts are made. From these result, it has been proposed that δ-ornithine acylase of KT 801 is at the cell surface.

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Concerning the δ-ornithine acylase, which hydrolyzes 5-N-acylornithine, the authors[1] studied on the distribution of the enzyme in soil bacteria, and observed extremely high δ-ornithine acylase activity in KT 801 (*Pseudomonas* sp.) which was isolated from soil. In a previous paper,[2] the authors studied on the cultural conditions and purification of the enzyme produced by KT 801, and obtained this bacterial δ-ornithine acylase as an electrophoretically and ultracentrifugally homogeneous protein.

The purpose of this report is to describe in detail some properties such as pH optima, ion requirement, heat stability, etc., and substrate Specificity of this new enzyme. On the other hand, concerning a location of δ-ornithine acylase in KT 801 cells, it was revealed that the enzyme is at the cell surface.

Materials and Methods

Preparation of Substrate

Of the substrates used for the present work, previously known compounds were prepared by established methods (see Table Ⅵ for references), characterized by their melting points and other properties, and all of which were in good agreement with those of literature.

ω-N-acyl derivatives of lysine, ornithine, and 2,4-diaminobutyric acid were prepared by the method of Kurtz.[3] Lysine, ornithine, or 2,4-diaminobutyric acid were converted to their copper salt with copper carbon-

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*3 This work was presented at the Meeting of the Kanto Division of the Pharmaceutical Society of Japan, Tokyo. December 24, 1966. This work is a part of the Ph. D. thesis of K. Matsui, University of Tokyo, M rch 1967.

1) Y. Kameda, K. Matsui: This Bulletin, **15**, 1573(1967).
2) K. Matsui, M. Kumagai, Y. Kameda: *Ibid.*, **15**, 1578(1967).
3) A.C. Kurtz: J. Biol. Chem., **180**, 1253 (1949).

ate, the salt solution were treated with the respective acid chlorides, and the copper were removed by hydrogen sulfide. The products were recrystallized from H_2O or H_2O·EtOH.

D-Ornithine was prepared by the hydrolysis of 2,5-di-N-benzoyl-D-ornithine which was prepared by assymmetric hydrolysis of 2,5-di-N-benzoyl-DL-ornithine with KT 218[4] (preserved bacteria in our laboratory) cell suspension. The procedure of assymmetric hydrolysis was essentially same described in previous paper.[5]

2-N-Acyl-L-ornithine were prepared by the method of Baldwin.[6] 5-N-carbobenzoxy-L-ornithine was acylated with acid chloride and the resulting 2-N-acyl-5-N-carbobenzoxy-L-ornithine was converted to 2-N-acyl-L-ornithine by catalytic hydrogenolysis.

5-N-Benzoyl-L-ornithine ethyl ester·HCl and 5-N-benzoyl-L-ornithine amide·HCl were prepared by the method of Izumiya, *et al.*,[7] which contain the esterification of 5-N-benzoyl-L-ornithine with anhydrous EtOH and HCl, and amidation of the ester with ammonia in MeOH. The analytical data and physical constants of these newly synthesized compounds are given in Table I.

Table I. Physical Constants and Analytical Data of New Compounds

New Compounds	m.p.	$[\alpha]_D$	Composition	Analysis (%) Calcd. C	H	N	Found C	H	N
5-N-*p*-Toluoyl-L-ornithine	244 (decomp.)	+23	$C_{13}H_{18}O_3N_2$	62.38	7.25	11.19	62.57	7.46	11.52
5-N-*m*-Nitrobenzoyl-L-Orn.	239 (decomp.)	+19	$C_{12}H_{15}O_5N_3$	51.24	5.38	14.94	51.26	5.54	14.60
5-N-*o*-Nitrobenzoyl-L-Orn.	211 (decomp.)	+19.5	$C_{12}H_{15}O_5N_3$	51.24	5.38	14.94	50.89	5.63	15.19
5-N-Phenylacetyl-L-Orn.	266 (decomp.)	+22	$C_{13}H_{18}O_3N_2$	62.38	7.25	11.19	62.09	7.35	11.22
5-N-Phenylacetyl-D-Orn.	266 (decomp.)	−21	$C_{13}H_{18}O_3N_2$	62.38	7.25	11.19	62.38	7.28	11.58
5-N-Phenylpropionyl-L-Orn.	250 (decomp.)	+22	$C_{14}H_{20}O_3N_2$	63.61	7.63	10.60	63.75	7.65	10.59
5-N-Cyclohexylcarbonyl-L-Orn.	240 (decomp.)	+21	$C_{12}H_{22}O_3N_2 \cdot \frac{1}{2}H_2O$	57.35	9.22	11.15	57.67	8.86	11.10
5-N-Cyclohexylacetyl-L-Orn.	250 (decomp.)	+23	$C_{13}H_{24}O_3N_2 \cdot \frac{1}{2}H_2O$	58.84	9.50	10.56	59.15	9.13	10.27
5-N-Hexanoyl-L-Orn.	252 (decomp.)	+22	$C_{11}H_{22}O_3N_2$	57.36	9.63	12.17	57.26	9.76	12.08
2-N-Phenylacetyl-L-Orn.	225 (decomp.)	+ 4	$C_{13}H_{18}O_3N_2$	62.38	7.25	11.19	62.36	7.52	11.46
2,5-Di-N-Phenylacetyl-L-Orn.	142	+ 4	$C_{21}H_{24}O_4N_2$	68.46	6.57	7.60	68.71	6.81	7.53
6-N-Phenylacetyl-L-lysine	263 (decomp.)	+14	$C_{14}H_{20}O_3N_2$	63.61	7.63	10.60	63.41	7.76	10.83
4-N-Phenylacetyl-L-2,4-diaminobutyric acid	230 (decomp.)	+21	$C_{12}H_{16}O_3N_2$	61.00	6.83	11.86	61.21	6.95	11.58

Used Enzyme

Purified δ-ornithine acylase obtained by the method described in previous paper[2] was appropriately diluted with 0.05*M* Tris buffer (pH 8.2) before use and used.

Enzyme Reaction

1) **Standard Enzyme Reaction**——Unless otherwise noted standard enzyme reaction was carried out as follows. The reaction mixture containing 1 ml. of 0.01*M* 5-N-benzoyl-L-ornithine and 0.5 ml. of 0.1*M* Tris buffer (pH 8.2) was preincubated at 37° for 5 min., after which 0.5 ml. of appropriately diluted enzyme solution was added and incubated at 37°. After 5 and 10 min., the liberated ornithine was measured by Chinard's acidic colorimetric ninhydrin method[8] with a minor modification.[1]

4) Y. Kameda, K. Matsui, Y. Kimura, E. Toyoura, M. Kimura : This Bulletin, **10**, 1146 (1962).
5) Y. Kameda, E. Toyoura, K. Matsui, Y. Kimura, Y. Kanaya, A. Nakatani, H. Saito, K. Kawase : Yakugaku Zasshi, **78**, 769 (1958).
6) B.C. Baldwin, D. Robinson, R.T. Williams : Biochem. J., **76**, 595 (1960).
7) N. Izumiya, H. Uchio, T. Kamata : Bull. Chem. Soc. Japan, **33**, 66 (1960).

That is, 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 boiling 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. The rate of hydrolysis was expressed in terms of micromoles of the substrate hydrolyzed per hr. per mg. of protein under the above conditions. Protein contents of the enzyme solution was determined by the method of Lowry, *et al.*[9] with crystalline bovine serum albumin as a standard.

2) Estimation of Hydrolytic Activity toward 5-N-Acylornithine or 2-N-Acylornithine——The method of estimation of hydrolytic activity toward 5-N-acyl or 2-N-acyl ornithine was completly the same with above described standard enzyme reaction.

3) Estimation of Hydrolytic Activity toward 6-N-Acyl-L-lysine——The method of estimation of hydrolytic activity toward 6-N-acyl-L-lysine was the same above described standard enzyme reaction without the last dilution.[1]

4) Estimation of Hydrolytic Activity toward 4-N-Acyl-L-2,4-diaminobutyric Acid——The liberated 2,4-diaminobutyric acid was measured by Work's acidic colorimetric ninhydrin method.[10] Above described reaction mixture were incubated at 37° and after 1 and 2 hr., 0.5 ml. of incubated mixture was added to 0.5 ml. of AcOH and 0.5 ml. of acidic ninhydrin reagent (ninhydrin is dissolved at 2% concentration in AcOH 6 ml. and 0.6*M* H_3PO_4 4 ml.), and the mixture was immersed in boiling water for 30 min. The solution was diluted with 3.5 ml. of AcOH and the absorption intensity was measured at 330 mμ, giving a measure of thef ree 2,4-diaminobutyric acid produced by the enzymatic hydrolysis.

5) Estimation of Hydrolytic Activity toward Other ω-Acylamino Acids——Digests consisting of 1 ml. of 0.01*M* substrate, 0.5 ml. of 0.1*M* Tris buffer (pH 8.2), and 0.5 ml. of enzyme solution were incubated at 37° and the liberated amino acid was measured by Moore and Stein's colorimetric ninhydrin method.[11] After 0, 10, and 20 min., 0.2 ml. of incubated mixture was added to a test tube which was preheated in boiling water and held at this temperature for 2 min. To these solution, 1 ml. of ninhydrin reagent (Dissolve 0.08 g. of $SnCl_2 \cdot 2H_2O$ in 50 ml. of the citrate buffer, pH 5.0. Add this solution to 2 g. of ninhydrin dissolved in 50 ml. of methyl cellosolve.) was added and the mixture was immersed in boiling water for 30 min. The solution was diluted with 5 ml. of 60% EtOH, and the absorption intensity after 5 fold dilution with 60% EtOH was measured at 570 mμ, giving a measure of the free ω-amino acid produced by the enzymatic hydrolysis.

6) Estimation of Hydrolytic Activity toward 5-N-Benzoyl-L-ornithine Ethyl Ester and 5-N-Benzoyl-L-ornithine Amide——Above described reaction mixture containing 5-N-benzoyl-L-ornithine ester or amide was incubated at 37° and after 0, 10, 20, and 30 min., 0.2 ml. of incubation mixture was added to a test tube which was preheated in boiling water and held at this temperature for 2 min. Estimation of ornithine ester or ornithine amide in these solution was made by paper chromatography. 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 in EtOH solution and colorized in an oven at 100° for 5 min. A spot of ornithine ester, ornithine amide, or ornithine were cut into species, placed into each test tube, added 1 ml. of ninhydrin reagents, and heated at 100° for 30 min. The solution were diluted with 5 ml. of 60% EtOH and the absorption intensity was measured at 570 mμ.

7) Estimation of Proteinase Activity[12]——Digests consisting 2.5 ml. of 0.6% casein in 0.1*M* Tris buffer (pH 8.2) and 0.5 ml. of enzyme solution were incubated at 37°. After 10 and 20 min., 2.5 ml. of 0.11*M* trichloroacetic acid was added and allowed to stand at 37° for 30 min. Then, the mixture was filtered and the absorption intensity of the filtrate was measured at 275 mμ.

A control solution was prepared as follows. 2.5 ml. of 0.6% casein was added to a mixture of 0.5 ml. of the enzyme solution and 25 ml. of 0.11*M* trichloroacetic acid and allowed to stand at 37° for 30 min. Then, the mixture was filtered and the filtrate was used for control.

Culture of KT 801 (*Pseudomonas* sp.)

Previously published method[2] was used for the culture of KT 801.

Preparation of Spheroplasts[13]

Cells of KT 801, 2 g. (wet weight), were routinely suspended in 150 ml. of sucrose-Tris medium (the mixture of 100 ml. of 20% sucrose and 50 ml. of 0.1*M* Tris buffer, pH 8.2) at 25°. 100 ml. of the suspension was supplemented with EDTA to a concentration of 0.001*M*, followed immediately by sufficient 1% lysozyme to give 10 μg. per ml. Timing of the reaction began at this point. The mixture was gently agitat-

8) F.P. Chinard : J. Biol. Chem., **199**, 91 (1952).
9) O.H. Lowry, N.J. Rosenbrough, A.L. Forr, R.T. Randall : *Ibid.*, **193**, 265 (1951).
10) E. Work : Biochem. J., **67**, 416 (1957).
11) S. Moore, W.H. Stein : J. Biol. Chem., **176**, 367 (1948).
12) S. Akabori : "Kōsokenkyuhō," **2**, 240 (1956).
13) M. Malamy, B.L. Horecker : Biochem. Biophys. Res. Comm., **5**, 104 (1961).

ed on a reciprocal shaker at 25°, and the extent of spheroplast formation was followed an optical density at 660 mμ after 10 fold dilution of the suspension in H_2O. At selected intervals, the suspension were centrifuged at 8,500 r.p.m. for 10 min. The resulting pellet was suspended in a volume of water equal to that of the original sucrose–Tris suspension. Vigorous mixing of the suspension caused lysis of the spheroplasts but not of any intact cells that may have remained, the latter were removed. Since the volume were equal, one may directly compare the concentration of enzyme in spheroplast lysate and in the sucrose–Tris medium surrounding the spheroplasts.

Cold Water Wash[14]

2 g. (wet weight) of cells was washed once with 0.01*M* Tris buffer (pH 8.2) and suspended in 150 ml. of sucrose–Tris medium (the mixture of 100 ml. of 20% sucrose and 50 ml. of 0.1*M* Tris buffer, pH 8.2).

100 ml. of the suspension was supplemented with EDTA to a concentration of 0.001*M*, gently agitated on a reciprocal shaker for 10 min. at 25°, and centrifuged at 10,000 r.p.m. for 10 min. The pellet was resuspended in 100 ml. of cold water ("cold water wash"), and after 10 min. of gentle agitation at 5°, the mixture was centrifuged at 10,000 r.p.m. for 10 min. The supernatant was called "cold water wash" and the pellet was suspended in 100 ml. of water. Since the volume were equal, one may directly compare the concentration of the enzyme in "cold water wash" and cell suspension.

Results and Discussion

Effects of pH on Activity and Stability

The effect of pH on the δ-ornithine acylase activity against 5-N-benzoyl-L-ornithine was determined over a pH range of 5.7 to 10.5 with phosphate, Tris-maleate, Tris, and carbonate buffers. As shown in Fig. 1, optimal pH is found at the neighborhood of 8.2, and the activity is considerably low below pH 7.0 or above pH 9.5. When the reaction in various buffers were compared, Tris, Tris-maleate, and phosphate buffer were found to be equally effective.

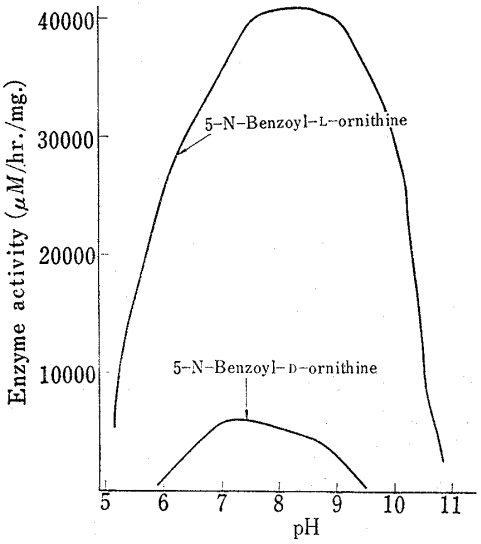

Fig. 1. Effect of pH on the Rate of Hydrolysis of 5-N-Benzoyl-L-ornithine and 5-N-Benzoyl-D-ornithine

Standard assay conditions are used.

Then, the effect of pH on the stability of δ-ornithine acylase was studied. Mixture of 1 ml. of the enzyme solution and 4 ml. of 0.05*M* each buffer were allowed to stand at 27° for several days and the enzyme activities were measured at pH 8.2. As shown in Table II, this enzyme is relatively stable in an alkaline pH region (8.0~10.0), but exposure to an acidic pH leads to severe inactivation.

Effect of Heat Treatment on the Enzyme Activity

A mixture of 1 ml. of enzyme solution and 1 ml. of 0.1*M* Tris buffer (pH 8.2) was heated at the indicated temperature. After 5 min., the mixture was rapidly chilled and the activity was measured. As shown in Table III, the activity loss is about 15% by treatment for 5 min. at 50° and the activity is completely lost by treatment for 5 min. at 70°.

Effect of Metal Ions, Chelating Agents, and Other Compounds

It has been well known that various α-amino acylases are specifically activated by Co^{2+} or Zn^{2+}.[15~18] Also it has been reported that ε-lysine acylase of KT 83 (*Pseudomonas* sp.) is activated by Zn^{2+}.[18]

14) H.C. Neu, L.A. Heppel : Biochem. Biophys. Res. Comm. **17**, 215 (1964).
15) R. Marshall, S.M. Birnbaum, J.P. Greenstein : J. Am. Chem. Soc., **78**, 4636 (1956).
16) R. Shirasaka : Ann. Takamine Lab., **10**, 74 (1958).
17) B.L. Vallee, H. Neurath : J. Am. Chem. Soc., **76**, 5006 (1954); J. Biol. Chem., **217**, 253 (1955).
18) S. Wada : J. Biochem., **46**, 445, 1541 (1959).

Table II. Effect of pH on the Stability of δ-Ornithine Acylase

pH and Buffer	Relative Activity[a]						
	5-N-Bz-L-Orn.					5-N-Bz-D-Orn.	
	0 hr.	24 hr.	72 hr.	96 hr.	120 hr.	0 hr.	72 hr.
6.0 Phosphate	100	63	10	0	0	100	11
7.0 Phosphate	100	75	33	11	2	100	37
8.0 Tris	100	102	103	97	95	100	100
9.0 Tris	100	100	102	98	97	100	95
9.5 Carbonate	100		100			100	100
10.0 Carbonate	100	98	102	97	87		
10.6 Carbonate	100		73			100	68

a) The activities toward 5-N-benzoyl-L-ornithine and 5-N-benzoyl-D-ornithine are respectively 41000 μ*M*/hr./mg. and 6500 μ*M*/hr./mg. at 0 hr. and these are taken 100.
Mixture of 1 ml. of enzyme solution and 4 ml. of 0.05*M* each buffer are allowed to stand at 37°. After indicated hr., 0.5 ml. of the mixture are taken out, diluted with 0.05*M* Tris buffer (pH 8.2 or 7.3) appropriately and measured the enzyme activities under standard conditions.

Table III. Effect of Heat Treatment on the Stability of δ-Ornithine Acylase

Heat Treatment	Relative Activity[a]		Heat Treatment	Relative Activity[a]	
	5-N-Bz-L-Orn.	5-N-Bz-D-Orn.		5-N-Bz-L-Orn.	5-N-Bz-D-Orn.
No Treatment	100	100	60° 5 min.	35	
50° 5 min.	85		65° 5 min.	3.5	
55° 5 min.	65	65	70° 5 min.	0	

a) Initial activities toward 5-N-benzoyl-L-ornithine and 5-N-benzoyl-D-ornithine are respectively 41000 μ*M*/hr./mg. and 6500 μ*M*/hr./mg. and these are taken 100.
A mixture of 1 ml. of enzyme solution and 1 ml. of 0.1*M* Tris buffer (pH 8.2) is heated at the indicated temperature. After 5 min., the mixture is rapidly chilled and the activities are measured under standard conditions.

In order to study the influence of metal ions on the δ-ornithine acylase activity, various metal ions were added to the enzyme assay. As shown in Table IV, Mg^{2+}, Mn^{2+}, Ca^{2+}, or Ba^{2+} have a slight stimulatory effect on the δ-ornithine acylase activity at $10^{-3}M$, but Co^{2+}, or Zn^{2+} have considerably inhibitory effect and Fe^{2+}, Fe^{3+}, Cu^{2+}, Hg^{2+}, or Ag^{+} have completely inhibited at a concentration $10^{-3}M$. These metal ions have no effect on the color reaction of ornithine.

Then the effect of chelating agents and other compounds on the activity was investigated. The sulfhydryl reagents such as *p*-chloromercuribenzoate has not affected to the enzyme activity at $5\times10^{-5}M$. Potassium cyanide and sodium azide show no inhibitory effect at $10^{-3}M$. Although great stimulatory effect have not been observed by addition of metal ions, the chelating agents such as EDTA or *o*-phenanthroline inhibit the enzyme activity. To elucidate this problem, EDTA was added to the enzyme solution at $10^{-2}M$ concentration. Then, the solution was dialyzed against 0.02*M* Tris buffer (pH 8.2) in order to remove the EDTA at 5° for 24 hr. The enzyme activity was depressed to 4% of initial activity by these process. To this enzyme solution, metal ions were added and the activities were measured. As shown in Table V, the enzyme activity was recovered to about 60% of the initial activity by addition of Mn^{2+} or Ca^{2+}. Since a decrease of the activity has been observed during the dialysis, it was supposed that the enzyme was considerably inactivated in that process. For this reason, it is elucidated that the recovery of the activity is not complete. From these results, it is suggested that δ-ornithine acylase is a metal enzyme and the binding affinity between the enzyme

Table IV. Effect of Metal Ions, Chelating Agents and Other Compounds on the δ-Ornithine Acylase Activity

Compounds	Relative Activity[a]			
	$4\times10^{-3}M$[b]	$10^{-3}M$	$2\times10^{-4}M$	$5\times10^{-5}M$
Mg^{2+}	111	107	107	
Mn^{2+}	107	109	104	
Ca^{2+}	109	109	104	
Ba^{2+}	109	107	99	
Ni^{2+}		89		
Co^{2+}		33		
Zn^{2+}		20		
Fe^{2+}		0		
Fe^{3+}		0		
Cu^{2+}		0		
Hg^{2+}		0		
Ag^{+}		0		
EDTA	49	65	71	
o-Phenanthroline	0	0	10	
Citrate		102		
Oxalate		102		
PCMB				107
KCN		94		
NaN_3		100		

a) The activity under no addition is taken as control (100).
b) Final concentration in reaction mixture.
The reaction mixture contains 1 ml. of 0.01*M* 5-N-benzoyl-L-ornithine, 0.5 ml. of appropriately diluted enzyme solution, and 0.5 ml. of 0.1*M* Tris buffer (pH 8.2) which contains the indicated test compounds. The activities are measured under standard conditions.

Table V. Effect of Chelating Agents and Metal Ions on δ-Ornithine Acylase Activity

	Relative[a] Activity		Relative[a] Activity
Enzyme Solution (Initial State)	100	Ca^{2+} $5\times10^{-4}M$	44
$10^{-2}M$ EDTA Added	17	$2\times10^{-3}M$	61
Dialysis to Tris Buffer (24 hr.)	4	Ba^{2+} $5\times10^{-4}M$	3.5
Mg^{2+} $5\times10^{-4}M$	18	$2\times10^{-3}M$	11
$2\times10^{-3}M$	29	$4\times10^{-4}M$ o-Phenanthroline Added	3.8
Mn^{2+} $5\times10^{-4}M$	49	Dialysis to Tris Buffer (24 hr.)	89
$2\times10^{-3}M$	57		

a) Initial activity is 41000 μ*M*/hr./mg. and this is taken 100.
EDTA is added to 5 ml. of the enzyme solution at $10^{-2}M$ concentration. Subsequently, this solution is dialyzed against 1 L. of 0.02*M* Tris buffer (pH 8.2) in order to remove the EDTA at 5° for 24 hr. and the activity is measured under the condition described in Table V.

protein and the metal is so firm that the metal can not be eliminated by the purification process. On the other hand, the inhibition for o-phenanthroline is completely recovered by the dialysis.

Effect of Substrate Concentration

The effect of substrate concentration on the activity of δ-ornithine acylase was investigated, and the results obtained have been plotted by the method of Lineweaver and Burk[19] for the estimation of Km. As shown in Fig. 2, Km was calculated to be 1.8

19) H. Lineweaver, D. Burk: J. Am. Chem. Soc., 56, 658 (1934).

$\times 10^{-4}M$ for 5-N-benzoyl-L-ornithine and the inhibition was observed in higher substrate concentration than $10^{-2}M$. On the other hand, reaction products such as benzoic acid and L-ornithine did not inhibit the enzyme activity under standard conditions.

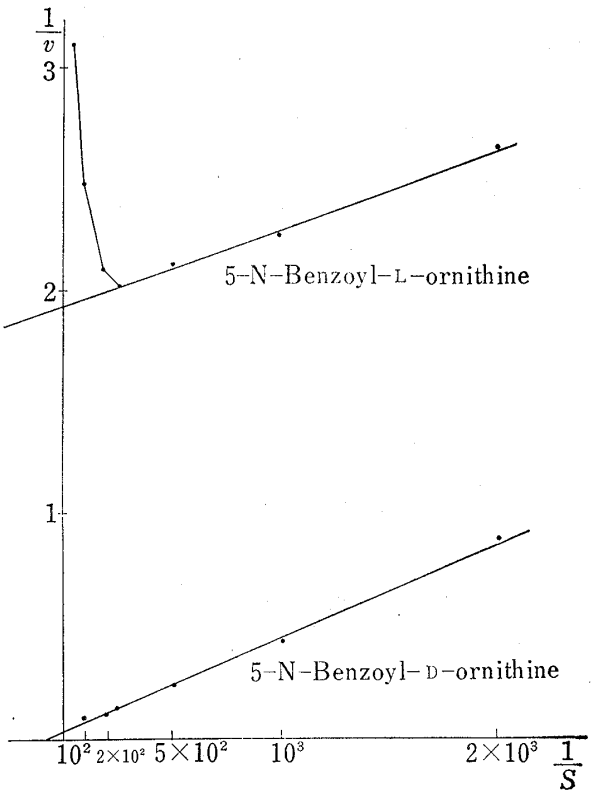

Fig. 2. Influence of 5-N-Benzoyl-L-ornithine and 5-N-Benzoyl-D-ornithine Concentration upon Velocity expressed as Reciprocals of Velocity and Substrate Concentration

The activity is measured under standard conditions except substrate concentration.

Substrate Specificity

Amino acylases are widely distributed in animals, plants, and microorganisms[20,21] and they have different substrate specificities. For example, hog kidney amino acylase I acts more readily on N-acylated aliphatic amino acids, whereas pancreatic carboxypeptidase acts more readily on N-acylated amino acids containing aromatic substituents on the β-carbon atom. In the case of ε-lysine acylases,[18,22] they can hydrolysis of 6-N-acyl-L-lysine, but not hydrolysis of 2-N-acyl-L-lysine or 5-N-acyl-L-ornithine. In order to clarify the substrate specificity of the δ-ornithine acylase, susceptibility of the various acyl derivatives of ornithine and its analogs toward the enzyme were investigated (Table VI).

1) Optical Specificity

δ-Ornithine acylase hydrolyses not only L form of 5-N-benzoylornithine, but D form of it in the rate of about one sixth.

2) Effect of Carboxyl and Amino Groups

5-N-Benzoyl-L-ornithine ethyl ester and 5-N-benzoyl-L-ornithine amide are not hydrolyzed by δ-ornithine acylase. These results suggest that susceptible substrate to δ-ornithine acylase requires absolutely the presence of a free carboxyl group. On the other hand, 5-benzamidovaleric acid, which has not α-amino group of ornithine, and 2,5-di-N-benzoyl-L-ornithine are hydrolyzed, although the rates of hydrolysis are very low. That is, the presence of a free α-amino group is desirable for ready susceptibility of the substrate, but it is not an absolute requirement as shown in the case of carboxyl group. From these results, it is suggested that δ-ornithine acylase hydrolyses both L and D form of 5-N-benzoylornithine.

3) Effect of Carbon Chain Length

As the homologues of 5-N-benzoyl-L-ornithine, the susceptibilities of 6-N-benzoyl-L-lysine and 4-N-benzoyl-L-2,4-diaminobutyric acid to the enzyme were studied. 6-N-Benzoyl-L-lysine was hydrolyzed by the enzyme at a rate of about one thirtieth of the ornithine derivative, but 4-N-benzoyl-L-2,4-diaminobutyric acid was not hydrolyzed at a measurable rate. A similar results were obtained in the case of ω-benzoylamino acid. 5-Benzamidovaleric acid which contains 5 carbon atoms is the most susceptible and 6-benzamidocaproic acid is followed. From these results, it is revealed that the

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Table Ⅵ. Substrate Specificity of δ-Ornithine Acylase

Compounds	Relative Activity	Reference
Optical Specificity		
5-N-Benzoyl-L-ornithine	100	23)
5-N-Benzoyl-D-ornithine	17	
Effect of Carboxyl and Amino Groups		
5-N-Benzoyl-L-ornithine ethyl ester	0	7)
5-N-Benzoyl-L-ornithine amide	0	7)
2,5-Di-N-benzoyl-L-ornithine	0.4	24)
2,5-Di-N-Phenylacetyl-L-ornithine	0	
5-Benzamido valeric acid	7	25)
Effect of Carbon Chain Length		
6-N-Benzoyl-L-lysine	3	3)
6-N-Phenylacetyl-L-lysine	0	
4-N-Benzoyl-L-2,4-diaminobutyric acid	0	3)
4-N-Phenylacetyl-L-2,4-diaminobutyric acid	0	
6-Benzamido caproic acid	0.6	26)
4-Benzamido butyric acid	0.06	27)
3-Benzamido propionic acid	0	28)
Effect of Acyl Group		
5-N-*p*-Toluoyl-L-ornithine	75	
5-N-*p*-Nitrobenzoyl-L-ornithine	21	23)
5-N-*m*-Nitrobenzoyl-L-ornithine	28	
5-N-*o*-Nitrobenzoyl-L-ornithine	0	
5-N-Phenylacetyl-L-ornithine	26	
5-N-Phenylpropionyl-L-ornithine	7.6	
5-N-Carbobenzoxy-L-ornithine	1.1	29)
5-N-Cyclohexylcarbonyl-L-ornithine	6.7	
5-N-Cyclohexylacetyl-L-ornithine	0.2	
5-N-*n*-Hexanoyl-L-ornithine	18.5	
5-N-Acetyl-L-ornithine	1.0	30)
5-N-Chloroacetyl-L-ornithine	8.0	31)
5-N-Benzenesulfonyl-L-ornithine	0	3)
L-Arginine (Amidino)	0	
L-Citrulline (Carbamoyl)	0	
α-Amino Acylase Activity		
2-N-Benzoyl-L-ornithine	0	6)
2-N-Phenylacetyl-L-ornithine	0	
Benzoyl-L-phenylalanine	0	32)
Benzoyl-D-phenylalanine	0	32)

The activity toward 5-N-benzoyl-L-ornithine is 41000 μ*M*/hr./mg. and this is taken as standard (100).

Digest consisting 1 ml. of 0.01*M* substrate, 0.5 ml. of 0.1*M* Tris buffer (pH 8.2), and 0.5 ml. of the enzyme solution is incubated at 37°. Activities are measured by the procedure described in "Method."

susceptibility of substrate to δ-ornithine acylase are considerably affected by carbon chain length between the susceptible carboxyamide group and free carboxyl group.

4) Effect of Acyl Groups

In order to test the effect of 5-N-acyl groups toward the δ-ornithine acylase activity, a number of 5-N-acyl-L-ornithine, indicated in Table Ⅵ, were prepared and suscep-

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tibilities to the enzyme were investigated at pH 8.2. The benzoyl derivative is the most susceptible in the tested compounds. The susceptibility is depressed by substitution of benzene nucleus with methyl or nitro groups. Particularly, 5-N-*o*-nitrobenzoyl-L-ornithine is not hydrolyzed by the enzyme. In addition, the rate of hydrolysis decrease by insertion of methylene group between the benzene nucleus and susceptible carboxyamide group such as phenylacetyl or phenylpropionyl derivatives. The susceptibilities of aliphatic acyl derivatives are generally low. Sulfonamide linkage such as 5-N-benzenesulfonyl-L-ornithine is not hydrolyzed. Also, arginine and citrulline ars not hydrolyzed.

On the other hand, it was reported that carboxypeptidase is specifically inhibited by the presence of phenylpropionic acid or phenylacetic acid.[33] Then, the effect of various organic acids, which were liberated by the hydrolysis of 5-N-acyl-L-ornithine, were investigated on the activity of δ-ornithine acylase. That is, the rate of hydrolysis of 5-N-benzoyl-L-ornithine was measured in the presence of each organic acids. As a results, the greater part of these organic acids have not inhibitory effect.

5) *α*-Amino Acylase Activity and Proteinase Activity

The δ-ornithine acylase has not α-amino acylase activities. Also, the δ-ornithine acylase has not proteinase activity, which is measured an increase of optical density at 275 mμ in trichloroacetic acid soluble solution, after incubation of casein with the enzyme.

Optimal pH toward 5-N-Benzoyl-D-ornithine

As shown in Table Ⅵ, δ-ornithine acylase hydrolyzes not only L form of 5-N-benzoyl ornithine, but D form. Then, pH optima toward 5-N-benzoyl-D-ornithine was studied for decide whether the pH optima is same or not. As shown in Fig. 1, shift of optimal pH is observed among the activities toward the D and L form of 5-N-benzoylornithine. The optimal pH against 5-N-benzoyl-L-ornithine is found at the neighborhood of 8.2 and the optimal pH against the D form is found at the neighborhood of 7.3. The activity toward the D form decreased 20% at pH 8.2 and 93% at pH 9.5, although the activity toward the L form decreased only 20% at pH 9.5. The both activities decreased below pH 7.0. It was reported that an optimal pH is different for the substrate in pepsin.[34] Then, the next experiments have been done for decide whether one enzyme hydrolyses L and D form of 5-N-benzoylornithine, or not.

1) Comparison of both activities in purification steps. As shown in Table Ⅶ, the ratio of the activities toward L and D form of 5-N-benzoylornithine remained constant all

Table Ⅶ. Optical Specificity of δ-Ornithine Acylase

Compounds	Relative Activity[a]		
	Cell Free Extract	$(NH_4)_2SO_4$ Fraction	Purified Enzyme
5-N-Benzoyl-L-ornithine	100	100	100
5-N-Benzoyl-D-ornithine	19	19	17
5-N-Phenylacetyl-L-ornithine	20	23	26
5-N-Phenylacetyl-D-ornithine	0.2	0.3	0.2

a) The activities toward 5-N-benzoyl-L-ornithine are 1500 μM/hr./mg. (cell free extract). 5700 μM/hr./mg. (ammonium sulfate fraction), and 41000 μM/hr./mg. (purified enzyme), and these are respectively taken as standard (100).
The activity toward 5-N-benzoyl-D-ornithine is measured at pH 7.3 and the activities toward other compounds are measured at pH 8.2 under standard conditions.

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34) F.A. Bovery, S.S. Yanari: "The Enzyme," **4**, 63 (1960). ed. by P.D. Boyer, H. Lardy, K. Myrback. Academic Press, Inc., Ltd.

through the purification steps. These results strongly suggest that one enzyme have the both activities.

2) Elution pattern of the both activities on DEAE cellulose chromatography. As shown in previous paper,[2] δ-ornithine acylase was purified by the chromatography on DEAE cellulose. Then, the both activities of effluents were measured and the elution pattern was shown in Fig. 3. The peak of the both activities overlaped one another and the ratio of the both activities remained constant in fraction 11 to 19.

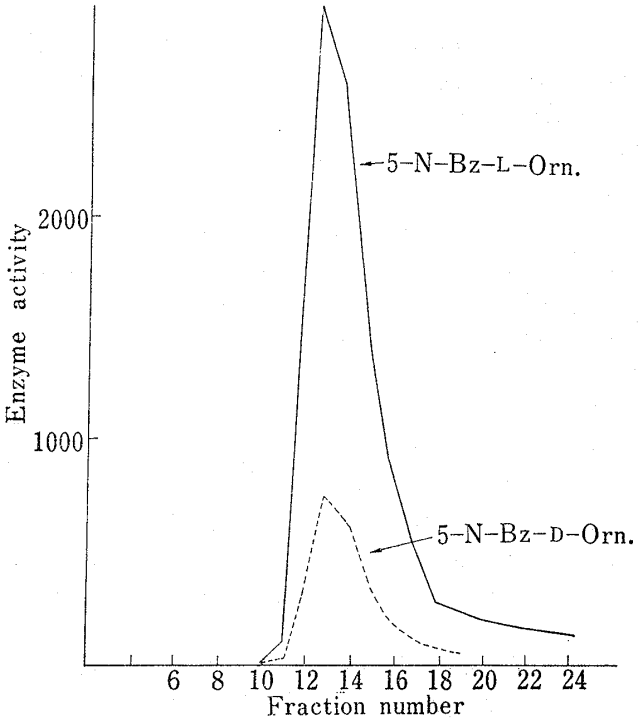

Fig. 3. Chromatography on DEAE Cellulose

The solid curve indicates enzyme activity (μ*M*/hr./ml.) toward 5-N-benzoyl-L-ornithine and dashed curve indicates enzyme activity toward 5-N-benzoyl-D-ornithine. Assays of the enzyme are done according to the standard procedure described in "Method."

3) Effect of pH on the enzyme stability. δ-Ornithine acylase is relatively stable in an alkaline pH region but is not stable in an acid pH region. On the other hand, it was observed that the activity toward 5-N-benzoyl-D-ornithine decrease at an alkaline pH region above pH 8.0. Then, the variation of the both activities were measured at respective optimal pH, after the mixture of 1 ml. of the enzyme solution and 1 ml. of 0.05*M* each buffer allowed to stand at 27° for 3 days. As shown in Table II, ratio of inactivation of the both activities are same in all pH region by the incubation.

4) Effect of heat treatment. A mixture of 1 ml. of the enzyme solution and 1 ml. of 0.1*M* Tris-maleate buffer (pH 7.3) was heated at 55°. After 5 min., the mixture was rapidly chilled and the both activities were measured at pH 7.3 or pH 8.2 after appropriate dilution. As shown in Table III, the both activities decreased respectively to 65% of the initial activities by this heat treatment.

5) Effect of metal ions and other compounds. Effect of metal ions or chelating agents etc. on the both activities were examined. As shown in Table VIII, *p*-chloromercuribenzoate and potassium cyanide have not effect to the both activities, and Co^{2+}, Fe^{3+}, Cu^{2+},

Table VIII. Effect of Metal Ions and Other Compounds on the Rate of Hydrolysis toward 5-N-Benzoyl-L-ornithine and 5-N-Benzoyl-D-ornithine

Added Compounds	Relative Activity		Added Compounds	Relative Activity	
	5-N-Bz-L-Orn.	5-N-Bz-D-Orn.		5-N-Bz-L-Orn.	5-N-Bz-D-Orn.
H_2O (Control)	100	100	Fe^{3+}	0	0
PCMB	98	95	Cu^{2+}	0	0
KCN	85	87	Hg^{2+}	0	0
Ca^{2+}	104	100	EDTA	45	35
Mn^{2+}	107	100	*o*-Phenanthroline	0	0
Co^{2+}	35	40			

The activities toward 5-N-benzoyl-D-ornithine was measured at pH 7.3. Other conditions and assay method are the same as those in Table IV.

Hg^{2+}, EDTA, and *o*-phenanthroline have the inhibitory effect toward the both activities in a same ratio.

From these results, it is suggested that one enzyme have the both activities. That is, δ-ornithine acylase has the hydrolytic activities toward L and D form of 5-N-benzoyl-ornithine, although the optimal pH of the both activities is different.

Michaelis Constant toward 5-N-Benzoyl-D-ornithine

Effect of 5-N-benzoyl-D-ornithine concentration on the activity of the δ-ornithine acylase was investigated, and the results obtained have been plotted by the method of Lineweaver and Burk[19] for the estimation of Km. As shown in Fig. 2, Km value was calculated to be $1.6\times10^{-2}M$ for 5-N-benzoyl-D-ornithine. That is, the Km value for D form is about 100 fold greater than the value for L form.

Localization of δ-Ornithine Acylase in KT 801 (*Pseudomonas* sp.)

It was reported by Pollock[35] that bacterial enzymes are divided into 3 groups according to their location in, on or around the cell: (1) truly intracellular, (2) surface-bound, (3) extracellular, and the most of extracellular enzyme, which contain polysaccharidase, mucopolysaccharidase, proteinase, peptidase, nuclease, phospholipase, lipase, penicillinase, etc. are hydrolytic enzymes. On the other hand, Malamy and Horecker[13] discovered that when *E. coli* cells were converted into spheroplasts by means of EDTA and lysozyme, the alkaline phosphatase was quantitatively released into the surrounding sucrose-Tris medium. In addition to this, Neu and Heppel[14] reported that a latent RNase, RNA-inhibited DNase, Co^{2+} stimulated 5′-nucleotidase, acid phosphatase and cyclic phosphodiesterase are set free by same procedure, and it is probably significant that all of them are degradative.

Then, the author examined the localization of δ-ornithine acylase in KT 801 cells. KT 801 cells were converted into spheroplasts by means of EDTA and lysozyme, and the activities in surrounding sucrose-Tris medium and spheroplasts lysate were measured. As shown in Table IX, δ-ornithine acylase was released into the surrounding sucrose-Tris medium by this treatment. But succinic dehydrogenase was not released in significant amount into the medium surrounding spheroplasts. The activity in spheroplasts supernatant is 45% of the original activity. But the activity is raised to 87% of the original activity by addition of Ca^{2+} at $2\times10^{-3}M$, since the δ-ornithine acylase activity is inhibited by the presence of EDTA and recovered by the addition of Ca^{2+} or Mn^{2+}. Neu and Heppel[14] reported that the alkaline phosphatase, cyclic phosphodiesterase, etc. are released by "cold water wash." As shown in Table IX, δ-ornithine acylase was also released by "cold water wash."

Table IX. Release of δ-Ornithine Acylase on Spheroplasts Formation and "Cold Water Wash"

	Activity μM/hr.	(Relative activity)
Cell suspension	19000	(100)
Spheroplasts suspernatant	8600	(45)
Spheroplasts suspernatant+$2\times10^{-3}M$ Ca^{2+}	16500	(87)
Spheroplasts lysate	1080	(5.6)
Spheroplasts lysate+$2\times10^{-3}M$ Ca^{2+}	1170	(6.2)
Cold water wash+$2\times10^{-3}M$ Ca^{2+}	14300	(75)
Cold water washed cell suspension	1140	(6)

Experimental procedures described in "Method."
All activities are expressed as enzyme units derived from 1.6 g. (wet weight) of cells.

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