Studies on the ϵ -Lysine Acylase

Part III*. A Surveys of the ϵ -Lysine Acylase Activity in Soil Organisms**

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Received October 9, 1961

In succession to previous papers, screening experiments for ϵ -lysine acylase activity in microorganisms from soils have been carried out. As a result, the enzyme activity was observed in some soil bacteria. From the organisms showing the activity, the strain which showed the highest activity was isolated and the microorganism was confirmed to belong in *Achromobacter pestifer*. So, further screening experiments of the typical organisms of *Achromobacter* and *Pseudomonas* species have been carried out, but the found activity in several organisms was much lower than that in *Achromobacter pestifer* strain EA isolated by the authors. ϵ -Lysine acylase in this organism shows optical specificity and hydrolyses only L-isomer but not D-isomer of ϵ -acetyllysine.

Acylases, which hydrolyse N-acyl amino acids, are classified into two groups. Namely, the first is α -amino acylase which hydrolyses α -N-acyl amino acids and the second is enzyme toward other N-acyl amino acids. With respect to α -amino acylase activities, the present authors have reported on the distribution and enzymatic properties of the activities in microorganisms¹⁻⁴⁾ and plants⁵⁾ besides in animals. On the other hand, Greenstein and his co-workers^{6,7)} first reported the occurrence of the ϵ -lysine acylase activity, that is, hydrolytic activity toward ϵ -N-acetyllysine, in rat tis-

sues, and Kameda and his co-workers⁸⁾ also found the activity in an organism of *Pseudomonas* obtained from soil.

In previous papers^{9,10)}, the authors reported on the screening experiments for ϵ -lysine acylase activity in the microorganisms and revealed the occurrence of the activity in molds, especially in *Aspergillus oryzae*. Although, this acylase is an induced enzyme whose activity is undetectable without induction and has no optical specificity. In other tested organisms the activities were scarcely found under the conditions employed by the authors.

However, it is quite possible that microorganisms possessing higher ϵ -lysine acylase activity occur in nature besides in the organisms previously mentioned. Therefore, in succession to the foregoing work, screening experiments in organisms isolated from soils and in typical bacteria of *Achromobacter* and

* Earlier reports by the present authors on the "Studies on Amino Acids. XIII⁹⁾ and XIV¹⁰⁾" are arranged as Part I and II of the serial reports of the "Studies on the ϵ -Lysine Acylase", respectively.

** Presented at the Meeting of the Kansai Division of the Agricultural Chemical Society of Japan, Kyoto, February 18, 1961.

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Pseudomonas species have been carried out by the same paper chromatographic method as employed in earlier experiment. As a result of present experiments, an organism showing extremely high ϵ -lysine acylase activity was isolated from soil and this organism was identified to belong in *Achromobacter pestifer*.

MATERIALS AND METHODS

Compounds Used. The preparations of ϵ -benzoyl-L-lysine, ϵ -benzoyl-D-lysine, ϵ -acetyl-L-lysine, acetyl-L-methionine and ϵ -benzoyl- α -acetyl-L-lysine were the same as previously described^{9,10}.

Organisms. Organisms in about 200 kinds of soils collected from all over the Japan, and 24 typical organisms of *Achromobacter* and *Pseudomonas* species used for the screening tests. As the sources of soil organisms, suspension of the soils in a saline solution was employed. The organisms of the genus *Achromobacter* and *Pseudomonas* were supplied from the Institute of Applied Microbiology, Tokyo University, and the Department of Fermentation Technology, Osaka University.

Media. Screening media employed were the same as used for the screening of bacteria and streptomycetes in a previous paper⁹. For preparation of enzyme solution, cultures were carried out in the media described in Table I.

TABLE I. COMPOSITION OF THE MEDIA FOR PREPARING ϵ -LYSINE ACYLASE

Media	Glucose (%)	Peptone (%)	Meat extract (%)	Minerals (%)	ϵ -Benzoyl-L-lysine (mM)
No. 1	—	1	1	NaCl	0.5
No. 2	1	1	1	NaCl	0.5
No. 3	5	1	—	$\left\{ \begin{array}{l} \text{KH}_2\text{PO}_4 \text{ 0.2} \\ \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \text{ 0.1} \end{array} \right.$	—

Assay of Enzyme Activity. In the screening experiments, enzyme assay method was the same as described in previous paper⁹. Unless otherwise noted, enzyme reaction was carried out by incubating a mixture of 1 ml of enzyme solution, 1 ml of 0.012 M ϵ -benzoyl-L- or -D-lysine and 1 ml of M/10 phosphate buffer (pH 7.0) at 37°C for appropriate hours. The liberated lysine was measured by Work's acidic colorimetric ninhydrin method with a minor modification as described in previous paper¹⁰. Enzyme activity was expressed in terms of micromoles of liberated lysine per hour per extracts of cells and filtered broth in a

unit ml of media.

Preparation of Enzyme Solution. Cell-free extracts and filtered broth were used as enzyme solution. Cell-free extracts were obtained in the following manner. The medium (100 ml each) in Table I was introduced to 500 ml shaking flasks and the cultures were incubated with reciprocal shaking (110 c/min, stroke 8 cm) at 27°C or 37°C for an appropriate period. The cells were harvested by centrifugation, washed twice with distilled water, ground with quartz sand and extracted with 100 ml of M/45 phosphate buffer (pH 7.0).

RESULTS

ϵ -Lysine Acylase Activity in Soil Organisms.

1. Screening Test. Microorganisms in about 200 kinds of soils were tested for ϵ -lysine acylase activities. Appearance of a distinct spot of lysine indicating the occurrence of ϵ -lysine acylase activity was observed in the organisms in about 10 kinds of soils. Besides the above organisms, low activities were recognized in the organisms in about 20 kinds of soils.

Screening for α -amino acylase activity was also carried out by the same method as in the previous paper⁹. Almost all organisms tested showed α -amino acylase activity. Namely, methionine spot was found in the broth of most organisms, although the spot of ϵ -benzoyllysine was scarcely detected.

2. Enzyme Activity. Since it was found from above experiments that the organisms in 10 kinds of soils showed ϵ -lysine acylase activity, the enzyme activities of these organisms were determined with cell-free extracts of 4 days culture employing No. 1 medium in Table I.

As shown in Table II, most of the soil organisms, which exhibited a lysine spot in above screening experiments, showed ϵ -lysine acylase activity also in cell-free extracts. The organisms in W445 o, collected at Nakamura, Kochi Prefecture, was found to have the highest enzyme activity among the tested organisms. Since the soil organisms tested

hydrolyzed only ϵ -benzoyl-L-lysine, but not ϵ -benzoyl-D-lysine, it is obvious that these acylase possessing activities have optical specificity.

3. **Identification of the Soil Organisms Producing the Most Potent ϵ -Lysine Acylase.** From the organisms in W445 o, which showed the highest enzyme activity in the above experi-

TABLE II. ϵ -LYSINE ACYLASE ACTIVITY PRODUCED BY SOIL ORGANISMS

Sources of Soil Organisms	ϵ -Lysine Acylase Activity*	
	ϵ -Benzoyl-L-lysine	ϵ -Benzoyl-D-lysine
W 430 a	0.022	0
W 415 b	0.017	0
W 331 a	0	0
W 416 h	0.040	0
W 403 i	0	0
W 436 b	0.019	0
W 411 h	0.017	0
W 445 o	2.12	0
W 115 c	0.017	0
W 445 g	0.032	0

* Enzyme activity was expressed in terms of micromoles of liberated lysine per hour per extracts of cells in a unit ml of media.

TABLE III. IDENTIFICATION OF THE BACTERIA PRODUCING ϵ -LYSINE ACYLASE

Size: $0.5 \sim 0.8 \times 1.0 \sim 2.0 \mu$.
 Shape: Rods.
 Spore: No forming.
 Gram: Negative.
 Flagella: Peritrichous.
 Motility: Weak motile.
 Gelatin: No liquefied.
 Milk: Unchanged.
 Litmus milk: Unchanged.
 Nitrate broth: Reaction positive.
 Methyl red test negative.
 Hydrogen sulfide not produced.
 Indole not produced.
 Citrate not utilized.
 No acid from glycerine, xylose, glucose, sucrose and starch.
 Optimum temperature: 30°C .
 Bouillon slant: Filiform, moderate, translucent, pale brown, medium unchanged.
 Bouillon colonies: Circular, entire, convex, butyrous, glistening, opaque brown, medium unchanged.
 Broth: Turbidic.

ments, the strain producing this activity was isolated by repeated plate cultures. Identification of this strain was kindly performed by Dr. H. Iizuka of the Institute of Applied Microbiology, Tokyo University, and the results are presented in Table III.

According to the Bergey's Manual of Determinative Bacteriology in the seventh editions, this strain was confirmed to belong in *Achromobacter pestifer*. Consequently, the authors designated the strain *Achromobacter pestifer* EA to distinguish it from the typical strain of *Achromobacter pestifer* supplied by the Institute of Applied Microbiology, Tokyo University, which shows only very low ϵ -lysine acylase activity as later described.

ϵ -Lysine Acylase Activity in Typical Organisms of *Achromobacter* and *Pseudomonas* Species.

1. **Screening Test.** From the results of the above identification and Kameda's study⁸⁾ on the soil bacteria producing ϵ -lysine acylase, the organisms producing high ϵ -lysine acylase activity were supposed to occur in the bacteria belonging in the genus *Achromobacter* and *Pseudomonas*. Therefore, by the same screening procedure as mentioned above, ϵ -lysine acylase activity in the 24 types of cultures of *Achromobacter* and *Pseudomonas* species was investigated.

The results, shown in Table IV, indicate that ϵ -lysine acylase activity occurs in some species of genus *Achromobacter* and *Pseudomonas*, but the enzyme activity was considerably lower compared with that of *Achromobacter pestifer* EA.

On the other hand, wide distribution of α -amino acylase activity was observed as in the previous report⁹⁾. Especially, the hydrolysis of acetylmethionine occurred in most of the organisms tested, but ϵ -benzoyl- α -acetyl-lysine was less susceptible.

2. **Enzyme Activity.** On the 6 organisms which exhibited a lysine spot in the above ex-

TABLE IV. ACYLASE ACTIVITIES IN *Achromobacter* AND *Pseudomonas* SPECIES

Organisms	Incuba- tion	ϵ -Lysine Acylase Activity								α -Amino Acylase Activity							
		Growth*				Detected Amino Acids**				Growth*				Detected Amino Acids**			
						Lysine		ϵ -Acetyl-lysine		ϵ -Benzoyl-lysine				Methio- nine		ϵ -Benzoyl-lysine	
		(days)		(days)		(days)		(days)		(days)		(days)		(days)		(days)	
	°C	2	5	2	5	2	5	2	5	2	5	2	5	2	5	2	5
<i>Achromobacter aquatilis</i>	30	+	+	—	—	■	■	■	■	+	+	+	■	±	+		
<i>Achromobacter candicans</i> 8005	"	+	■	—	±	■	■	■	■	+	+	—	—	—	—		
<i>Achromobacter convexum</i>	"	+	+	—	—	■	■	■	■	+	■	■	■	—	—		
<i>Achromobacter dendriticum</i>	"	±	±	—	—	■	■	■	■	±	+	+	■	—	—		
<i>Achromobacter hartlebii</i>	"	+	■	—	—	■	■	■	■	±	+	■	■	±	+		
<i>Achromobacter liquidum</i>	"	±	+	—	—	■	■	■	■	+	+	■	■	±	+		
" var <i>miyamizu</i>	"	+	+	—	—	■	■	■	■	+	+	■	■	±	+		
<i>Achromobacter nadaense</i>	"	+	+	—	+	■	■	■	■	±	±	■	■	—	±		
<i>Achromobacter eurydice</i>	"	■	■	—	—	■	■	■	■	+	+	—	—	—	—		
<i>Achromobacter superficial</i>	"	■	■	—	±	■	■	■	■	■	■	■	■	—	—		
<i>Achromobacter pestifer</i>	"	■	■	—	±	■	■	■	■	+	■	■	■	—	±		
" EA***	"	■	■	■	■	■	■	+	—	+	+	■	■	—	—		
<i>Achromobacter cycloclastes</i>	"	+	+	—	■	■	■	■	■	+	+	+	■	—	±		
<i>Achromobacter delmarvae</i>	"	+	■	—	—	■	■	■	■	+	■	—	■	—	—		
<i>Pseudomonas fluorescens</i>	"	■	■	—	—	■	■	■	■	■	■	■	■	—	+		
<i>Pseudomonas gelidicola</i>	"	+	+	—	—	■	■	■	■	±	■	±	■	—	+		
<i>Pseudomonas aeruginosa</i> 8229	37	■	■	—	±	■	■	■	■	■	■	■	■	—	—		
" 8230	"	■	■	—	—	■	■	■	■	■	■	+	■	—	—		
" 8231	"	■	■	—	—	■	■	■	■	■	■	+	■	—	—		
" 8252	"	■	■	±	+	■	■	■	■	■	■	■	■	—	±		
<i>Pseudomonas frari</i>	30	+	+	—	—	■	■	■	■	+	■	+	■	—	±		
<i>Pseudomonas taetrolens</i>	"	+	■	—	—	■	■	■	■	+	■	±	+	—	—		
<i>Pseudomonas striafaciens</i>	"	■	■	—	—	■	■	■	■	±	+	±	■	—	—		
<i>Pseudomonas ribuliflava</i>	"	■	■	+	■	■	■	■	■	+	+	+	■	—	—		

* + Indicates the relative amounts of growth.

** + Indicates the relative intensity of the spots on the paper chromatogram, ■ corresponds approximately to the intensity of the spot of 0.05 M amino acid.

*** Isolated from soil by the authors.

periment, enzyme activity was examined with cell-free extracts and filtered broth.

As shown in Table V, almost all organisms tested produced the enzyme in the media employed, but the activities of these organisms were much lower than that of *Achromobacter pestifer* EA. It is of interest to note that the typical strain of *Achromobacter pestifer* shows only very slight activity.

Optical Specificity of ϵ -Lysine Acylase in *Achromobacter pestifer* EA.

From the above experiments it was confirm-

ed that ϵ -lysine acylase in *Achromobacter pestifer* EA shows very high activity comparing with other microorganisms. So, to ascertain whether the ϵ -lysine acylase can be used for the resolution of racemic lysine or not, optical specificity of this enzyme was further investigated.

The results, shown in Table VI, indicate that the enzyme hydrolyses only ϵ -benzoyl-L-lysine, but not ϵ -benzoyl-D-lysine at any pH. The highest activity for hydrolysis of ϵ -benzoyl-L-lysine was found to be at pH 5.0.

TABLE V. ϵ -LYSINE ACYLASE IN SOME SPECIES OF THE GENUS *Achromobacter* AND *Pseudomonas*

Organisms	Media	No. 1		No. 2			No. 3		
		72	120	24	48	72	24	48	120
<i>Achromobacter nadoense</i>	Cell-free extract	0	0	0	0	0	0	0	0
	Broth	0.01	0	0	0	0	0	0	0
<i>Achromobacter cycloclastes</i>	Cell-free extract	0.02	0.01	0.15	0.07	0.04	0.07	0.08	0.01
	Broth	0	0	0.05	0.04	0.03	0.02	0.02	0.01
<i>Achromobacter pestifer</i>	Cell-free extract	0.01	—	0.07	—	0.04	0.05	—	0.02
	Broth	0	—	0	—	0	0	—	0
<i>Achromobacter pestifer</i> EA	Cell-free extract	79.3	—	95.9	108.8	—	103.1	144.4	—
	Broth	19.5	—	29.2	67.4	—	25.3	35.7	—
<i>Pseudomonas aeruginosa</i> 8229	Cell-free extract	0	0	0.03	0.05	0	0.02	0	0
	Broth	0.01	0	0	0	0	0	0	0
<i>Pseudomonas aeruginosa</i> 8252	Cell-free extract	0	0.04	0.03	0.02	0	0	0	0
	Broth	0	0	0	0	0	0	0	0
<i>Pseudomonas riboflavina</i>	Cell-free extract	2.98	0.41	1.76	1.55	0	0.90	0.99	0
	Broth	0.06	0.08	0.01	0.01	0.27	0	0	0

Enzyme activity was expressed in terms of micromoles of liberated lysine per hour per cell-free extracts and filtered broth in a unit ml of media.

TABLE VI. OPTICAL SPECIFICITY OF ϵ -LYSINE ACYLASE IN *Achromobacter pestifer* EA

Substrates	Reaction pH	3.6	5.0	7.0	8.9
ϵ -Benzoyl-L-lysine	41*	160	99	64	
ϵ -Benzoyl-D-lysine	0	0	0	0	

* Micromoles of liberated lysine per hour per extracts of cells in a unit ml of media.

Cultivation was carried out for 24 hrs at 30°C using No. 3 medium in Table I. Below pH 5.0, M/10 acetate buffer was used and above pH 7.0, M/10 phosphate buffer was used.

DISCUSSION

Until the publication of the authors' previous papers^{9,10}, occurrence of ϵ -lysine acylase activity was reported only in animal tissues and in an organism obtained from soil. The authors carried out the investigation on the distribution of the activity in microorganisms for the purpose of finding out advantageous enzyme sources for optical resolution of DL-lysine. As a result, in previous paper⁹ the authors first disclosed the occurrence of the activity in molds, especially in *Aspergillus oryzae*, and the study of the enzymatic properties revealed that the enzyme activity in mold is inducibly formed under the presence of ϵ -benzoyllysine and has no optical specificity on the hydrolysis of ϵ -benzoyl-DL-lysine.

In succession to the previous report, screen-

ing experiments for ϵ -lysine acylase in soil bacteria were performed. As the result of the present study, occurrence of the ϵ -lysine acylase activity in some soil bacteria was found and confirmed by the experiments employing cell-free extracts of these bacteria. From the soil organisms showing the activity, the authors isolated the strain which shows the highest activity. By the identification, this strain was confirmed to belong in *Achromobacter pestifer* as described in Bergey's Manual and the authors designated the strain *Achromobacter pestifer* EA.

From the above experiments, further screening for ϵ -lysine acylase in the typical organisms of *Achromobacter* and *Pseudomonas* species has been carried out. Although the activity was found in several species, it is very low compared with that in *Achromobacter pestifer* EA. Hydrolytic activity in typical strain of *Achromobacter pestifer* was also very slight. Consequently, the extraordinary high ϵ -lysine acylase activity in *Achromobacter pestifer* EA is a specific nature of this strain and not a common properties of the organisms in the genus *Pseudomonas* and *Achromobacter*.

Differing from the ϵ -lysine acylase activity

in *Aspergillus oryzae*, the activity in *Achromobacter pestifer* EA shows optical specificity and only acts upon the L-isomer of ϵ -benzoyl-lysine. This indicates the usefulness of this organisms as the enzyme source for the optical resolution of ϵ -benzoyl-DL-lysine.

On the screening studies for α -amino acylase, occurrence of the activity was observed in almost all microorganisms tested. From the previous and present studies, it is apparent that α -amino acylase activity occurs widely among microorganisms. Although ϵ -ben-

zoyl- α -acetyllysine is less susceptible to α -amino acylase, acetylmethionine is readily hydrolyzed by most microorganisms.

Acknowledgements. The authors are grateful to Prof. H. Mitsuda of Kyoto University, Dr. M. Fujisawa and Dr. N. Sugimoto of this Laboratory for their helpful advice and encouragement. They are also indebted to Assist. Prof. H. Iizuka of Institute of Applied Microbiology, Tokyo University, for identification of the organism in this work.