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Studies on Acylase Activity and Micro-organisms. XXVII.[1] Purification and Properties of Phenylacetyl DL-Acylase (N-Phenylacetyl-DL-amino-acid Amidohydrolase) from *AAA* 6020 (*Pseudomonas* sp.)

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Screening experiment in soil bacteria have been carried out for the purpose of finding out new acylases. As a result, *AAA* 6020 (*Pseudomonas* sp.) which produces a new acylase (tentatively called phenylacetyl-DL-acylase) was isolated. *AAA* 6020 was incubated in the synthetic medium containing of phenylacetyl-D-leucine and the bacterial cells were harvested. The phenylacetyl-DL-acylase was extracted from *AAA* 6020 by means of sonic oscillation and purified by ammonium sulfate fractionation, DEAE cellulose chromatography and Sephadex G-200 gelfiltration.

The purified enzyme was represented about 29 fold purification over the cell free extract and the specific activity toward N-phenylacetyl-D-leucine was 124 units/mg. The homogeneity of purified enzyme was demonstrated by means of disc electrophoresis and analytical ultracentrifugation. A molecular weight of the enzyme was estimated to be about 115000 by gelfiltration. The optimal pH toward N-phenylacetyl-D-leucine was 8.0. This enzyme hydrolysed not only N-phenylacetyl-D-amino acids but also N-phenylacetyl-L-amino acids. However N-benzoyl and N-acetyl derivatives of D- and L-amino acids were not hydrolyzed. Thus this DL-acylase has specificity toward phenylacetyl-amino acids but has not optical specificity.

Keywords——DL-acylase; N-phenylacetyl-DL-amino acid amidohydrolase; DL-acylase purification; substrate specificity; soil bacteria; *Pseudomonas*

In a previous paper,[1] the authors reported that the D-acylase of *AAA* 6029 (*Pseudomonas* sp.) can hydrolyze N-acyl derivatives of D-amino acid but can not hydrolyze N-acyl-L-amino acids. The D-acylase of *AAA* 6029 hydrolyzes N-benzoyl-D-amino acids more readily than N-phenylacetyl-D-amino acids.

During the course of investigation on D-acylase in this laboratory, screening experiments for D-acylase activity in soil bacteria have been carried out for the purpose of finding out new D-acylases. The present investigation was undertaken to find an enzyme which hydrolyzes N-phenylacetyl-D-amino acids. A number of bacteria was cultured on the medium containing phenylacetyl-D-amino acids, and their culture brothes were examined for the liberated amino acids by paper chromatography. As a result the authors found that *AAA* 6020 (*Pseudomonas* sp.) produces a new type of acylase which hydrolyzes not only N-phenylacetyl-D-amino acids but N-phenylacetyl-L-amino acids. The purpose of this report is to describe in detail the purification of the enzyme and its properties.

Materials and Methods

Preparation of Substrate——Substrate used for the present work were preparated by established method,[3] characterized by their melting points and other properties, and of which were in good agreement with those of literature.

1) Part XXVI: Y. Kameda, T. Hase, S. Kanatomo, and Y. Kita, *Chem. Pharm. Bull.* (Tokyo), **26**, 2698 (1978).
2) Location: *3 Ho, Kanagawa-machi, Kanazawa 920–11, Japan*; *a*) To whom inquire of this paper should be addressed; *b*) *13–1, Takara-machi, Kanazawa 920, Japan.*
3) T. Suyama, T. Toyota, and S. Kanao, *Yakugaku Zasshi*, **85**, 279 (1965).

Isolation of *AAA* 6020 Producing DL-Acylase——*AAA* 6020 capable of utilizing N-phenylacetyl-D-threonine as a sole source of carbon was isolated by enrichment culture from the soil sample obtained from the herbal garden of Kanazawa University. Elective culture of microorganisms from soil was carried out aerobically at 27° in test tube with a basal medium of the following composition. Carbon source (N-phenylacetyl-D-threonine), 0.1 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). A loop of the culture fluid was planted in an agar medium, containing 1.5% agar in the basal medium. Culture experiments were carried out in order to determine whether the microbe isolated from the agar plate could grow in the basal medium.

Identification of *AAA* 6020——The soil bacteria *AAA* 6020 was identified on the basis of the described method.[4]

Assay of Enzyme Activity——1) D-Acylase Activity: The reaction mixture, containing 50 μl of 0.05 M N-phenylacetyl-D-leucine, 100 μl of 0.1 M Tris buffer (pH 8.0), and 50 μl of appropriately diluted enzyme solution was incubated at 37°. After 15 and 30 min, the reaction was terminated by adding 200 μl of 12% trichloroacetic acid. The mixture was allowed to stand for about 30 min and centrifuged at 3000 *g* for 15 min. The amount of amino acid liberated by the reaction was determined by the ninhydrin method as described previous paper.[5] One unit of acylase is defined as that amount of enzyme which renders one micromole of liberated amino acid in 1 hr under the above condition.

2) L-Acylase Activity: L-Acylase activity was estimated in 0.1 M phosphate buffer (pH 7.0). Other conditions and method were completely the same with above described method.

Protein Determination——Protein concentration was determined by the method of Warburg, *et al.*[6] with absorbance at 260 and 280 nm, and the method of Lowry, *et al.*[7] with bovine serum albumin as standard.

Cultivation——100 ml of seed culture of the bacteria was inoculated to 900 ml of basal medium containing N-phenylacetyl-D-valine as a sole source of carbon, and was cultivated at 27° for 3 days with reciprocal shaking. The bacteria was harvested by centrifugation (20000 *g*, 15 min) and washed with cold 0.9% NaCl.

Extraction of DL-Acylase from *AAA* 6020——The bacterial pastes (21.6 g) obtained from 9 liters of the above culture fluid were suspended in 80 ml of 0.05 M Tris buffer and ruptured with sonic oscillation (Tomy Model UR 2000P) under ice cooling. The sonicated suspension was centrifuged at 32000 *g* for 15 min, and the supernatant was collected.

Purification of DL-Acylase of *AAA* 6020——All steps in the purification were carried out at 4° in 0.05 M Tris buffer (pH 8.0). Solid $(NH_4)_2SO_4$ was added to the cell free extract and the precipitate between 20 and 50% saturation was collected by centrifugation at 20000 *g* for 15 min. The precipitate was suspended in cold 0.05 M Tris buffer (pH 8.0) and the suspension was subsequently dialyzed against the same buffer, which was replaced periodically. The dialyzed enzyme solution was applied to a column of DEAE cellulose (3.6 × 40 cm), which had been equilibrated with the buffer. The column was washed with the buffer and the adsorbed proteins were eluted with 2000 ml of the same buffer with linearly increasing NaCl concentration from 0 to 0.3 M. The fraction were collected in 20 ml portion at a flow rate of 120 ml/hr. Active fraction was concentrated by ultrafiltration. The concentrated enzyme solution was applied to a column of Sephadex G-200 (2.6 × 90 cm) which had been equilibrated with the buffer. The fractions were collected in 20 ml portion at a flow rate of 15 ml/hr. The active fraction was applied to a column of DEAE cellulose (2.2 × 33 cm), washed with the buffer, and eluted with increasing linear gradient of NaCl (0 to 0.3 M) contained in the buffer. The fractions were collected in 10 ml portion at a flow rate of 100 ml/hr. The active fraction was concentrated by ultrafiltration. The concentrated enzyme solution was applied to a column of Sephadex G-200 (2.6 × 90 cm), and fractions were collected in 10 ml portion at a flow rate of 15 ml/hr. The active fraction was used in all experiments for examination of enzymatic properties.

Ultracentrifugal Analysis——Sedimentation velocity analysis was carried out with a Spinco Model E Analytical Ultracentrifuge Operating at 996 rps at 20°.

Disc Electrophoresis——Polyacryl amide disc gel electrophoresis was carried out according to the method of Nagai[8] using pH 8.3 gel and a current of 3 mA per tube was applied for 0.5—1 hr. Gel was stained with Amidoblack dye and destained by gently shaking in frequent changes of 7% acetic acid over 48 hr.

Results and Discussion

Identification of *AAA* 6020

AAA 6020 was obtained from a soil sample by using the medium containing N-phenylacetyl-D-threonine as a sole source of carbon. It was Gram negative straight rods, 0.7 μ×

4) M. Doudroff and N.J. Palleroni, "Bergey's Mannual of Determinative Bacteriology," 8th ed., ed. by R.E. Buchanan and N.E. Gibbons, The Williams and Wilkins Company, Baltimore, 1974, p. 217; "Saikingaku Jisshu Teiyo," 5 ed., ed. by Tokyo University Ikagakukenkyusho Gakuyukai, 1976.
5) Y. Kameda, T. Kuramoto, D. Matsui, and T. Ebara, *Chem. Pharm. Bull.* (Tokyo), **16**, 1023 (1968).
6) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1941).
7) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
8) Y. Nagai, *Tanpakushitsu Kakusan Koso*, **11**, 744, 817 (1966).

2.1 μ, and had polar flagella (Electron microscopy photograph, negative staining by phosphotungstic acid). Taxonomic nature of *AAA* 6020 was summarized in Table I. According to the Bergy's Manual Determinative Bacteriology,[4] this strain was seemed to belong in *Pseudomonas* group.

Table I. Taxonomic Nature of *AAA* 6020 (*Pseudomonas* sp.)

Shape: Rods	Methylred test: Negative
Size: 0.7 μ × 2.1 μ	Gelatin, Starch } Not liquefied
Flagella: Polar flagella	
Gram: Negative	Glucose-ammonium soln.
Motility: Possessed	Glucose-nitrate soln.
Glucose: Metabolized by oxidation	Succinate-nitrate soln. } Utilized
Oxidase reaction: Positive	Glutamate soln.
Catalase reaction: Positive	*p*-Hydroxybenzoate soln.
Indole: Not produced	Growth at 4° or 42° in bouillon: Not grew
Nitrites: Produced from nitrates	

Metabolic Activities of *AAA* 6020

Metabolic activities of *AAA* 6020 on acyl D-amino acids, phenylacetic acid, and benzoic acid were studied and the results were shown in Table II. *AAA* 6020 metabolized phenylacetic acid, benzoic acid and phenylacetyl-D-amino acids but did not metabolize benzoyl-D-amino acids. Next D-acylase activity of the bacteria was tested by paper chromatography of the culture broth. Paper chromatography was carried out with the ascending method using Toyo filterpaper No. 51 and BuOH: AcOH: H_2O=4: 1: 2 as a solvent. After development, the paper chromatogram was treated with 0.3% ninhydrin–EtOH solution and estimated a corresponding spot of amino acid. *AAA* 6020 seemed to possess D-acylase activity which hydrolyze N-phenylacetyl-D-amino acids but not N-benzoyl-D-amino acids.

Table II. Metabolic Activity of *AAA* 6020

$C_6H_5CH_2COOH$	+	C_6H_5COOH	+
$C_6H_5CH_2CO$-D-Leu	+	C_6H_5CO-D-Leu	−
$C_6H_5CH_2CO$-D-Met	+	C_6H_5CO-D-Met	−
$C_6H_5CH_2CO$-D-Thr	+	C_6H_5CO-D-Thr	−
$C_6H_5CH_2CO$-D-Val	+	C_6H_5CO-D-Val	−
$C_6H_5CH_2CO$-D-Phe	±	C_6H_5CO-D-Phe	−

+: Within 4 days at 27°, luxuriant growth of bacteria on a culture medium was observed.
−: Growth of bacteria was not observed.

Effect of Culture Medium on the Production of DL-Acylase

Since it is known that an amount of enzyme in microorganism is induced by the specific substance, the authors investigated effect of culture medium on the production of DL-acylase. D-Acylase activity was produced when *AAA* 6020 had been grown in synthetic medium containing phenylacetyl amino acids such as phenylacetyl-D-leucine, phenylacetyl-D-valine, phenylacetyl-D-phenylalanine, and corresponding phenylacetyl-L-amino acids. However, the D-acylase activity was not produced when the bacteria had been grown in bouillon or synthetic medium containing phenylacetic acid as a sole source of carbon. The best medium for production of DL-acylase among the tested medium was the medium containing phenylacetyl-D-valine. Thus the medium containing 0.1% phenylacetyl-D-valine, 0.1% NH_4Cl, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 1 ml/liter of 1% $CaCl_2$, 0.5 ml/liter of $FeCl_3$, (pH 7.2) was used for the production of the enzyme.

Purification of DL-Acylase

DL-Acylase was purified by ammonium sulfate fractionation, DEAE cellulose chromatography, Sephadex G-200 gelfiltration, 2nd DEAE cellulose chromatography, and 2nd Sephadex G-200 gelfiltration. The elution pattern of 2nd Sephadex G-200 gelfiltration was shown in Fig. 1. The D-acylase activity in the fraction 27—32 of 2nd Sephadex G-200 gel filtration was combined and used as "purified enzyme" for the following studies. The specific activity of this enzyme toward N-phenylacetyl-D-leucine was 124 units/mg and represented about 29 fold purification over the cell free extract. A summary of a typical purification is presented in Table III.

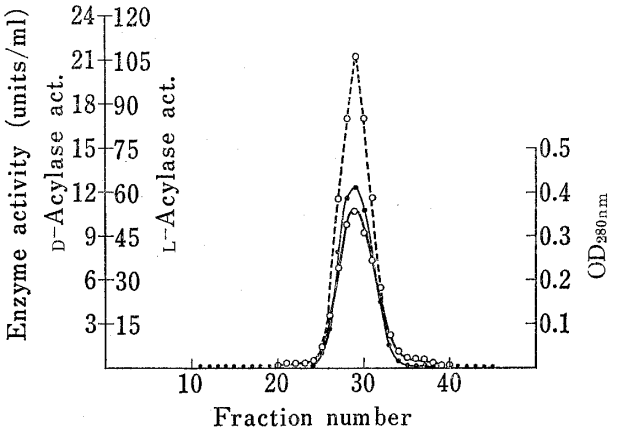

Fig. 1. Sephadex G-200 Gelfiltration

D-Acylase activity toward phenylacetyl-D-leucine is indicated by (—○—). L-Acylase activity toward phenylacetyl-L-leucine is indicated by (---○---). Protein concentration measured at 280 nm is indicated by (—●—).

5.6 ml of enzyme solution was applied to Sephadex G-200 column (2.6×90 cm). The flow rate was 15 ml/hr and 10 ml fractions were collected.

TABLE III. Purification of DL-Acylase of *AAA* 6020

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification
Cell suspension	3170	9820	3.1		
Cell free extract	1930	8490	4.3	100	1
$(NH_4)_2SO_4$ fractionation	1053	6600	6.2	78	1.4
DEAE cellulose	169	4280	25.2	50	5.9
Sephadex G-200	50.9	2900	57.1	34	13.3
2nd DEAE cellulose	23.0	2280	98.8	27	23.0
2nd Sephadex G-200	13.8	1690	124.0	20	29.0

The enzyme activity was measured toward N-phenylacetyl-D-leucine (Standard enzyme reaction).
The protein concentration was measured using Lowry method.

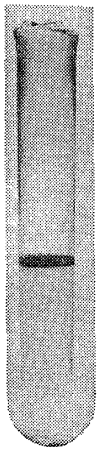

Fig. 2. Disc Electrophoretic Analysis of the Purified DL-Acylase

The protein migrated from the top toward the bottom of the gel.

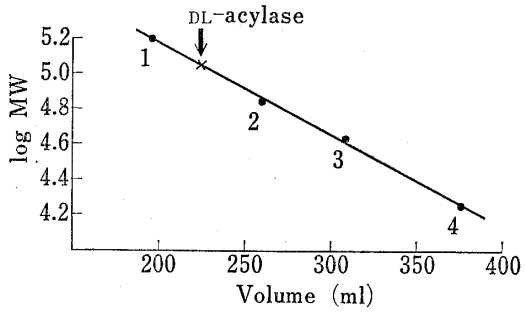

Fig. 3. Determination of the Molecular Weight of the DL-Acylase by Gelfiltration of Sephadex G-200

column size: 2.6×90 cm.
buffer: 0.05 M tris buffer (pH 8.0).
1: gamma globulin; 2: albumin; 3: obalbumin; 4: myoglobin.

Purity

Purity of the purified enzyme preparation was examined by ultracentrifugal and disc electrophoretic analysis. A single peak was observed throughout the ultracentrifugal run and the sedimentation coefficient was 7.8 S. A single protein band was also obtained in disc electrophoretic analysis at pH 8.3 gel (Fig. 2).

Molecular Weight

The molecular weight of DL-acylase was determined by using the method of Andrews[9] by gelfiltration of Sephadex G-200. As shown in Fig. 3, the molecular weight was calculated to be about 115000 for the enzyme.

Substrate Specificity of DL-Acylase

α-Amino acylase are widely distributed in animals, plants, and microorganism[10] and they have different substrate specificities. For example, hog kidney amino acylase I acts more readily on N-acylated aliphatic L-amino acid, whereas pancreatic carboxypeptidase acts more readily on N-acylated L-amino acids containing aromatic substituents on the β-carbon atom. In order to clarify the substrate specificity of the DL-acylase, susceptibility of the various acyl derivatives of amino acids toward the enzyme was investigated.

1) **Effect of Acyl Group on D-Acylase Activity**——As shown in Table IV, N-phenylacetyl derivatives of D-leucine, D-glutamic acid, D-alanine, D-threonine, D-methionine, D-valine, and D-phenylalanine were hydrolyzed by this acylase. But it did not hydrolyzed N-benzoyl derivatives of D-amino acids or N-acetyl derivatives of D-amino acids at a measurable rate. From these results, it is revealed the susceptibility of substrate are considerably affected by acyl groups.

2) **Effect of Amino Acid on D-Acylase Activity**——As shown in Table IV, the susceptivility was decreased in order, phenylacetyl-D-leucine>phenylacetyl-D-glutamic acid>phenyl acetyl-D-alanine>phenylacetyl-D-threonine>phenylacetyl-D-methionine>phenylacetyl-D-valine≧phenylacetyl-D-phenylalanine.

Table IV. Substrate Specificity of D-Acylase Activity of DL-Acylase

	Phenylacetyl derivatives	Benzoyl derivatives	Acetyl derivatives
D-Leu	100	0	0
D-Glu	82	0	
D-Ala	58	0	
D-Thr	42	0	
D-Met	27	0	
D-Val	13	0	
D-Phe	12	0	0

The activity toward phenylacetyl-D-leucine is 124 units/mg and this is taken 100.
Digest consisting 50 μl of 0.05 M substrate, 100 μl of 0.1 M Tris buffer (pH 8.0), and 50 μl of the enzyme solution is incubated at 37°. Activities are measured by the procedure in "Method."

3) **Optical Specificity of DL-Acylase**——As shown in Table IV and V this α-amino acylase hydrolyzed not only phenylacetyl-D-amino acids but also phenylacetyl-L-amino acids, and it hydrolyzed phenylacetyl-L-amino acids more readily than phenylacetyl-D-amino acids.

9) P. Andrews, *Biochem. J.*, **96**, 595 (1965).
10) J.P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 2, John Wiley and Sons, Inc. 1961, p. 1754; I. Chibata and T. Tosa, *Bull. Agr. Chem. Soc. Japan*, **23**, 370 (1959).

4) **L-Acylase Activity of DL-Acylase**——As shown in Table V this DL-acylase hydrolyzed N-phenylacetyl-L-amino acids but not N-benzoyl-L-amino acids or N-acetyl-L-amino acids. Effect of amino acid for susceptibility of phenylacetyl-amino acid to the L-acylase activity was different to the D-acylase activity, and the susceptibility followed to phenylacetyl-L-valine, phenylacetyl-L-alanine, phenylacetyl-L-glutamic acid, phenylacetyl-L-methionine, phenylacetyl-L-leucine, phenylacetyl-L-phenylalanine. In the case of *AAA* 6029[1] D-acylase was separated from L-acylase, so the question had been raised whether a few of α-amino acylases might be contaminated in this enzyme preparation and one of them might hydrolyze phenylacetyl-D-amino acids and the another might hydrolyze phenylacetyl-L-amino acids. To make clear the question, optimal pH, pH stability, effect of metal ions and other compounds, and thermal stability of both activities were measured.

TABLE V. Substrate Specificity of L-Acylase Activity of DL-Acylase

	Phenylacetyl derivatives	Benzoyl derivatives	Acetyl derivatives
L-Val	1227	0	
L-Ala	1188	0	
L-Glu	765	0	
L-Met	727	0	
L-Leu	695	0	0
L-Phe	627	0	0
L-Tyr	587		
L-Asp	473	0	
L-Trp	400		
D-Leu	100		

The activity toward phenylacetyl-D-leucine is 124 units/mg and this is taken 100.

Digest consisting 50 μl of 0.05 M substrate, 100 μl of 0.1 M phosphate buffer (pH 7.0), and 50 μl of the enzyme solution is incubated at 37°. Activities are measured by the procedure described in "Method."

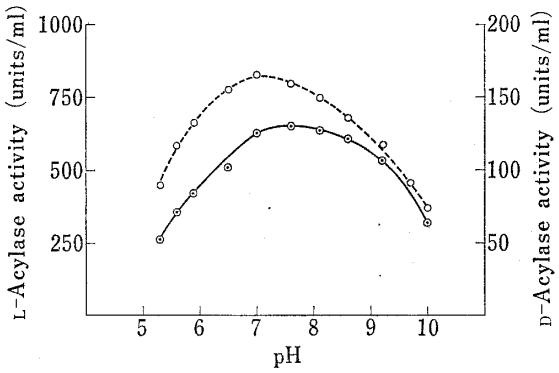

Fig. 4. Effect of pH on D-Acylase Activity and L- Acylase Activity

—○—: D-acylase activity toward phenylacetyl-D-leucine.
---○---: L-acylase activity toward phenylacetyl-L-leucine.
Activities are measured by the procedure described in "Method."

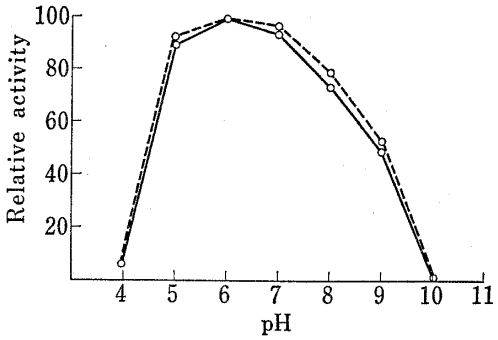

Fig. 5. Effect of pH on the Stability of D-Acylase Activity and L-Acylase Activity

—○—: D-acylase activity toward phenylacetyl-D-leucine.
---○---: L-acylase activity toward phenylacetyl-L-leucine.
Remaining activities are measured by the procedure described in "Method."

Effect of pH on Activity and Stability

The effect of pH on D- and L-acylase activities against N-phenylacetyl-D-leucine and N-phenylacetyl-L-leucine was measured over a pH of 5.0—11.0 with acetate, phosphate, Tris, and borate buffer. As shown in Fig. 4, optimal pH against N-phenylacetyl-D-leucine was found at neighborhood of 8.0 and optimal pH against N-phenylacetyl-L-leucine was found at neighborhood of 7.0. The effect of pH on stability of DL-acylase was studied. The mixture of 1 ml of the enzyme solution and 4 ml of 0.05 M buffers of various pH values were allowed to stand at 37° for 1 hr. As shown in Fig. 5, the enzyme was relatively stable at pH 5—7 but it was unstable at below 5.0 or above 7.0. However, in routine storage at 4°, the enzyme was apparently stable at pH 6—8 for months. Ratio of stability of the activities toward N-phenylacetyl-D-leucine and N-phenylacetyl-L-leucine were same in all pH region.

Thermal Stability of DL-Acylase

A mixture of 1 ml of the enzyme solution and 1 ml of 0.1 M Tris buffer (pH 8.0) was heated at the indicated temperature. After 5 min, the mixture was rapidly chilled and the activity was measured. As shown in Fig. 6, both D- and L-acylase activities were about 10% by treatment for 5 min at 40° and these activities were completely lost by treatment for 5 min at 50°. Ratio of inactivation of the activities toward N-phenylacetyl-D-leucine and N-phenylacetyl-L-leucine were same by this heat treatment.

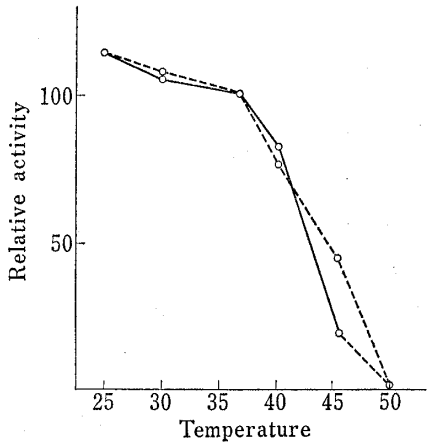

Fig. 6. Thermal Stability of D-Acylase Activity and L-Acylase Activity

—○—: D-acylase activity toward phenylacetyl-D-leucine.
---○---: L-acylase activity toward phenylacetyl-L-leucine.
Remaining activities are measured by the procedure described in "Method."

Table VI. Effect of Metal Ions, Chelating Agents, and Other Compounds on D-Acylase Activity and L-Acylase Activity

Addition[a]	D-Acylase activity[b]	L-Acylase activity[b]
None	100[c]	100[c]
L-Ascorbic acid	102	103
β-Mercaptoethanol	96	94
H_2O_2	107	105
PCMB	100	102
o-Phenanthroline	99	92
EDTA	93	102
$CoCl_2$	87	87
$FeSO_4$	102	98
$MgCl_2$	86	92
$CaCl_2$	89	97
$MnCl_2$	98	94
$CuSO_4$	102	107
$HgCl_2$	74	76
$ZnSO_4$	64	80

a) The reaction mixture contains 50 μl of 0.05 M phenylacetyl-D-leucine or phenylacetyl-L-leucine, 50 μl of appropriately diluted enzyme solution, and 100 μl of 0.1 M Tris buffer (pH 8.0) which contains the indicated test compounds. Final concentration of added compounds is 1×10^{-3} M. The activity are measured as described in "Method."
b) D-Acylase activity was measured toward phenylacetyl-D-leucine. L-Acylase activity was measured toward phenylacetyl-L-leucine.
c) The activity under no addition is taken as control (100).

Effect of Metal Ions, Chelating Agents, and Other Compounds

It has been well known that various α-amino acylases are specially activated by Co^{2+} or Zn^{2+}.[11] The D-acylase of *AAA* 6029 is inactivated by Hg^{2+} and Zn^{2+}. The influence of metal

11) R. Marshall, S.M. Birnbaum, and J.P. Greenstein, *J. Am. Chem. Soc.*, **78**, 4636 (1956); B.L. Vallee and H. Neurath, *J. Biol. Chem.*, **217**, 253 (1955).

ions on D- and L-acylase activities toward N-phenylacetyl-D-leucine and N-phenylacetyl-L-leucine were examined. As shown in Table VI, Hg^{2+}, Zn^{2+}, and Co^{2+} had a slight inhibitory effect on both acylase activities at 10^{-3} M. But other ions had not effect to both activities at 10^{-3} M. The effect of chelating agents and other compounds are studied on the both activities.[12] *p*-Chloromercuribenzoate, L-ascorbic acid, β-mercaptoethanol, and hydrogen peroxide had not affected to both enzyme activities at 10^{-3} M. The chelating agents such as EDTA or *o*-phenanthroline did not affect both activities.

Ratio of D- and L-Acylase Activities in Purification Steps

1) The ratio of activities toward N-phenylacetyl-D-leucine and N-phenylacetyl-L-leucine in purification steps were measured. As shown in Table VII, the ratio of the activities toward L and D form of N-phenylacetyl-leucine remained constant all through purification steps.

Table VII. Ratio of D-Acylase Activity and L-Acylase Activity in Purification Steps

	Cell suspension	Cell free extract	$(NH_4)_2SO_4$ fractionation	DEAE cellulose	Sephadex G-200	Hydroxyl apatite	2nd DEAE cellulose	2nd Sephadex G-200
$\frac{\text{L-Acylase activity}}{\text{D-Acylase activity}}$	5.8	6.7	6.8	6.9	6.6	7.0	7.2	7.0

D-Acylase activity is measured toward N-phenylacetyl-D-leucine. L-Acylase activity is measured toward N-phenylacetyl-L-leucine. The activity are measured by the procedure described in "Method".

2) When elution pattern of the both D- and L-acylase activities on DEAE cellulose chromatography and Sephadex G-200 gelfiltration were measured, the peak of the both activities overlapped one another and the ratio of the both activities remained constant in all fractions. These results suggested that one enzyme has both D-acylase and L-acylase activities.

Effect of Substrate Concentration

The effect of substrate concentration on the activities of DL-acylase were investigated by the method of Linweaver and Burk.[13] The K_m value were calculated to be 5.6×10^{-3} M for N-phenylacetyl-D-leucine and to be 7.1×10^{4} M for N-phenylacetyl-L-leucine. The K_m value for D form is about 8 fold greater than the value for L-form.

The authors previously reported that the L-acylase of *KT* 801[5] (*Pseudomonas* sp.) can hydrolyze N-benzoyl-L-amino acids but not N-phenylacetyl-L-amino acids. On the contrary, the L-acylase of *E. coli K*-12 recombinante[12] hydrolyzes N-phenylacetyl-L-amino acids but not N-benzoyl-L-amino acids. The D-acylase of *AAA* 6029 (*Pseudomonas* sp.) hydrolyzes N-acyl-D-amino acids but not N-acyl-L-amino acids, and it hydrolyzes N-benzoyl-D-amino acids more readily than N-phenylacetyl-D-amino acids. Newly obtained the DL-acylase of *AAA* 6020 can hydrolyze not only N-phenylacetyl-D-amino acids but also N-phenylacetyl-L-amino acids, however it can not hydrolyze N-benzoyl or N-acetyl derivatives of amino acids. Thus this DL-acylase has a specificity toward N-phenylacetyl-amino acid but has not optical specificity.

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12) Y. Kameda and T. Iwahi, presented at the 24th Meeting of the Pharmaceutical Society of Japan, Kyoto, April, 1967.
13) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).