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Studies on Acylase Activity and Micro-organisms. XXVI.[1] Purification and Properties of D-Acylase (N-Acyl-D-amino-acid Amidohydrolase) from *AAA* 6029 (*Pseudomonas* sp.)[2]

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AAA 6029 (*Pseudomonas* sp.) was isolated from soil by using the synthetic medium containing N-benzoyl-D-phenylalanine as a sole source of carbon. The bacteria produce a D-acylase which hydrolyzes N-acyl-D-amino acids. The D-acylase was extracted by means of sonic oscillation and purified by ammonium sulfate fractionation, DEAE cellulose chromatography, and Sephadex G-100 gelfiltration. The purified enzyme was represented about 900 fold purification over the cell free extract. The molecular weight of this enzyme was estimated to be about 45000 by gelfiltration. This enzyme can hydrolyze N-benzoyl and N-acetyl derivatives of the D-form of phenylalanine, methionine, leucine, alanine, and valine. But the acylase can not hydrolyze N-acyl derivatives of L-amino acids.

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

In work reported earlier,[4] it was shown that a strain of *Pseudomonas* sp., *KT* 83, can hydrolyze N-benzoyl derivatives of both L- and D-forms of amino-acids such as phenylalanine, tyrosine and alanine. In a further experiment[5] carried out with the *KT* 83 acetone powder, it hydrolyzed N-dichloroacetylated D-isomers of *threo-β*-phenylserine, *threo-β-p*-nitrophenylserine and phenylglycine more easily than the corresponding N-dichloroacetylated L-isomers, suggesting the possible occurrence of D-acylase in this bacteria species. Next the occurrence of D-acylase was demonstrated by the cell free extract of *KT* 83.[6] Then, the authors had been investigating on the purification of D-acylase in *KT* 83, but had unfortunately lost the strain of *KT* 83. Therefore, the authors screened a number of soil bacteria in order to find out new D-acylase. As a result, a strain (tentatively called *AAA* 6029) which produce D-acylase was isolated. This strain was seemed to belong in *Pseudomonas* group. The purpose of this report is to described in detail the purification of the D-acylase and its properties.

Materials and Methods

Preparation of Substrates——Substrates used for the present work were prepared by described method.[7] N-Formyl-D-phenylalanine, N-formyl-L-phenylalanine, and N-formyl-glycine were purchased from Tokyo-kasei Co.

Isolation of Soil Bacteria *AAA* 6029 Capable of Utilizing N-Benzoyl-D-phenylalanine——The constituents of the culture medium used for the isolation of *AAA* 6029 were as follows: Benzoyl-D-phenylalanine, 0.1 g;

1) Part XXV: Y. Kameda, T. Kuramoto, K. Matsui, and T. Ebara, *Chem. Pharm. Bull.* (Tokyo), **16**, 1023 (1968).
2) A part of this work was reported at the 48th Annual Meeting of the Japanese Biochemical Society, Fukuoka, October, (1975).
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4) Y. Kameda, E. Toyoura, H. Yamazoe, Y. Kimura, and Y. Yasuda, *Nature*, **170**, 888 (1952).
5) Y. Kameda, E. Toyoura, Y. Kimura, and K. Matsui, *Yakugaku Zasshi*, **78**, 202 (1958).
6) Y. Kameda, E. Toyoura, and Y. Kimura, *Nature*, **181**, 1225 (1958).
7) T. Suyama, T. Toyota, and S. Kanao, *Yakugaku Zasshi*, **85**, 279 (1965).

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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 soil sample (0.3 g) taken from the herbal garden of Kanazawa University was inoculated into 10 ml of the above culture medium and incubated at 27° for 5 days. When luxuriant growth of bacteria occurred, the bacteria *AAA* 6029 was selected in conventional method. The stock culture of *AAA* 6029 was maintained in N-benzoyl-D-phenylalanine medium and subcultured at weekly intervals.

Identification of the Bacteria——The soil bacteria *AAA* 6029 was identified on the basis of the described method.[8]

Assay of Enzyme Activity——1) Standard Enzyme Reaction: The reaction mixture, containing 50 μl of 0.05 M N-benzoyl-D-phenylalanine, 100 μl of 0.1 M carbonate buffer (pH 10.5), and 50 μl of appropriately diluted enzyme solution was incubated at 30°. After 15 and 30 min, the reaction was terminated by adding of 200 μl of 12% trichloroacetic acid. The mixture was allowed to stand for about 30 min and centrifuged at 3000 rpm for 15 min. The amount of amino acid liberated by the reaction was determined by the ninhydrin method.[9] 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) Hydrolytic Activity toward N-Benzoyl, N-Acetyl, N-Formyl, and N-Phenylacetyl Derivatives of D-Amino Acid: A buffer for reaction mixture was used carbonate (pH 10.5) toward N-benzoyl-D-amino acid; Tris-malate (pH 6.8) toward N-acetyl-D-amino acid and N-formyl-D-amino acid; Tris-HCl (pH 8.0) toward N-phenylacetyl-D-amino acid. Other condition and method were the same with above described method.

3) L-Acylase Activity: As optimal pH of L-acylase activity toward N-benzoyl-L-phenylalanine was found at neighborhood of 7.0, L-acylase activity toward N-acyl-L-amino acid was measured in 0.1 M Tris-malate buffer (pH 6.8), and other condition was the same with above described standard condition.

Estimation of Protein——Protein concentration was determined by the method of Warburg *et al.*[10] with absorbance at 260 and 280 nm, and the method of Lowry *et al.*[11] with bovine serum albumin as standard.

Growth of Bacteria and Extraction of D-Acylase——One liter of the seed culture of *AAA* 6029 was inoculated to 9 l of the culture medium described above and cultivation was carried out at 27° with reciprocal shaking (130 cycle/min, stroke 7 cm) for 3 days. The bacterial cells were harvested by centrifugation at 20000 ***g*** for 15 min and washed with cold 0.9% NaCl. Buffer A (0.01 M Tris-HCl buffer, pH 8.0, containing 10% (v/v) glycerol) was used for enzyme preparation. The bacterial pastes (22.4 g) obtained from 9 liters of the above culture fluid were suspended in 80 ml of ice-cold buffer A and disrupted by ultrasonic treatment (Tomy Model UR 2000P) under ice cooling. The ruptured cells was centrifuged at 32000 ***g***, 4° for 15 min and the supernatant fraction was collected.

Purification of D-Acylase——All steps in the purification were carried out at 4° in the buffer A. Solid $(NH_4)_2SO_4$ was added to the cell free extract and precipitates between 20 and 50% saturation were collected by centrifugation at 20000 ***g*** for 15 min. The precipitate was suspended in cold buffer A and was subsequently dialyzed against the buffer A, which was replaced periodically. The dialyzed enzyme solution was applied to a column of DEAE cellulose (3.6×43 cm) which had been equilibrated with the buffer A. The column was washed with the buffer A and the adsorbed proteins were eluted with 2000 ml of the buffer A with linearly increasing NaCl concentration from 0 to 0.5 M. The fractions were collected in 20 ml portion at a flow rate of 120 ml/hr. The active fraction was concentrated by ultrafiltration. The concentrated enzyme solution was applied to a column of Sephadex G-100 (2.6×96 cm) which had been equilibrated with buffer A. 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 (1.6×32 cm), washed with buffer A, and eluted with increasing linear gradient of NaCl (0 to 0.5 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-100 (2.6×96 cm) and fractions were collected in 5.8 ml portion at a flow rate of 15 ml/hr. The active fractions were combined and used in all experiments for examination of enzymatic properties.

Disc Electrophoresis——Polyacryl amide disc gel electrophoresis was carried out according to the method of Nagai[12] using pH 8.3 gel. Sample containing about 200 μg of protein was loaded on 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.

8) 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.
9) Y. Kameda, T. Kuramoto, K. Matsui, and T. Ebara, *Chem. Pharm. Bull.* (Tokyo), **16**, 1023 (1968).
10) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1941).
11) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
12) Y. Nagai, *Tanpakushitsu Kakusan Koso*, **11**, 744, 818 (1966).

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Results and Discussion

Identification of the Strain (*AAA* 6029)

AAA 6029 was isolated from a soil by use of a synthetic medium containing N-benzoyl-D-phenylalanine as a sole source of carbon. It was Gram negative straight rods, 0.7—0.8 μ ×1.9—2.3 μ, and had polar flagella (electron microscopy photograph, shadowing by Pt/Pd =4/1). The taxonomic nature of *AAA* 6029 was summarized in Table I. According to the Bergy's Manual Determinative Bacteriology,[8] this strain was confirmed to belong in *Pseudomonas* group.

Table I. Taxonomic Nature of *AAA* 6029 (*Pseudomonas* sp.) Producing D-Acylase

Shape: Rods	Nitrites: Produced from nitrates
Size: 0.7—0.8 μ × 1.9—2.3 μ	Indole: Not produced
Flagella: Polar flagella	Starch } Liquefied
Gram: Negative	Gelatine } Liquefied
Motility: Possessed	Glucose-ammonium soln. } Utilized
Glucose: Metabolized by oxidation	Glucose-nitrate soln. } Utilized
Oxidase reaction: Positive	Succinate-nitrate soln. } Utilized
Catalase reaction: Positive	*p*-Hydroxylbenzoate soln. } Utilized
Fluorescent pigments: Produced in culture medium	Growth at 42° and at 5°: Negative

Purification of D-Acylase of *AAA* 6029

D-Acylase of *AAA* 6029 was purified by ammonium sulfate fractionation, DEAE cellulose chromatography, Sephadex G-100 gel filtration, 2nd DEAE cellulose chromatography, and 2nd Sephadex G-100 gel filtration. The elution pattern of 1st DEAE cellulose chromatography was shown in Fig. 1. D-Acylase activity toward N-benzoyl-D-phenylalanine was eluted in about 0.15 M NaCl, and L-acylase activity was eluted in about 0.4 M NaCl. L-Acylase which had been present 416 units in the cell free extract and 336 units in the ammonium sulfate fractionation was removed from D-acylase in this step. Elution pattern of 2nd

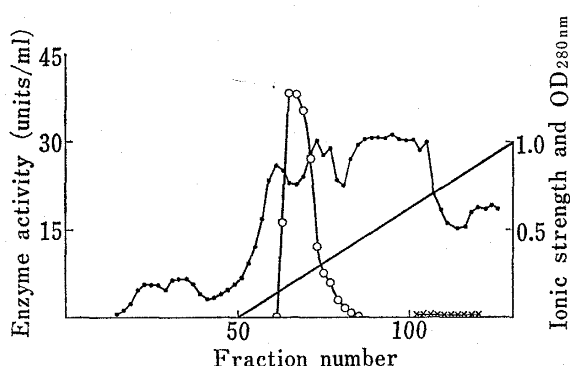

Fig. 1. Chromatography on DEAE Cellulose

D-Acylase activity toward N-benzoyl-D-phenylalanine is indicated by (—○—). L-Acylase activity toward N-benzoyl-L-phenylalanine is indicated by (—×—). Protein concentration measured at 280 nm is indicated by (—•—). Ionic strength gradient is indicated by (——). Assays of enzyme were done according to the standard method described in "Method."

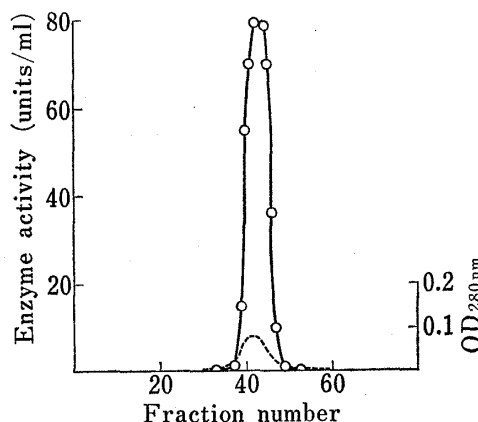

Fig. 2. Sephadex G-100 Gelfiltration

6.4 ml of enzyme solution was applied to Sephadex G-100 column (2.6×96 cm). The flow rate was 15 ml/hr and 5.8 ml fractions were collected. D-Acylase activity is indicated by (—○—). Protein concentration measured at OD_{280nm} is indicated by (--------).

Sephadex G-100 gel filtration was shown in Fig. 2. The D-acylase activity in the fraction 41—45 of 2nd Sephadex G-100 gel filtration were combined and used as "purified enzyme" for the following studies. The specific activity of this enzyme solution was 2437 units/mg

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toward N-benzoyl-D-phenylalanine and represented about 900 fold purification over the cell free extract. A summary of a typical purification is presented in Table II. This D-acylase was labile and its purification was carried out as rapidly as possible in order to minimize the loss of activity.

TABLE II. Purification of D-Acylase of *AAA* 6029

Purification step	Volume ml	Protein[a)] mg	Activity[b)] u	Specific activity u/mg	Recovery %	Purification
Cell free extract	231	5221	14100	2.7	100	1
$(NH_4)_2SO_4$ fraction	145	1399	11600	8.3	82	3.1
1st DEAE cellulose	213	151	7900	52.4	56	19.4
1st Sephadex G-100	57	15	7290	486	51	180
2nd DEAE cellulose	28	5.6	5230	935	37	346
2nd Sephadex G-100	27	1.7	4240	2437	30	902

a) The protein concentration was measured using Lowry method.
b) The enzyme activity was measured using N-benzoyl-D-phenylalanine as substrate (standard enzyme reaction).

Purity

Purity of the purified enzyme preparation was examined by disc electrophoresis at pH 8.3. Two minor components were observed besides a main band.

Molecular Weight

The molecular weight of the D-acylase was determined using the method of Andrews[13)] by gelfiltration on Sephadex G-100. As shown in Fig. 3, the molecular weight was calculated to be about 45000 for the D-acylase.

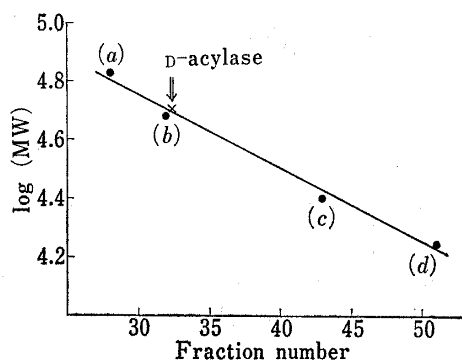

Fig. 3. Molecular Weight Determination of the D-Acylase by Sephadex G-100 Gelfiltration

The standard proteins used for calibration were bovine serum albumin (*a*), ovalbumin (*b*), chymotrypsinogen (*c*), and myoglobin (*d*).
Column size: 2.6×96 cm.

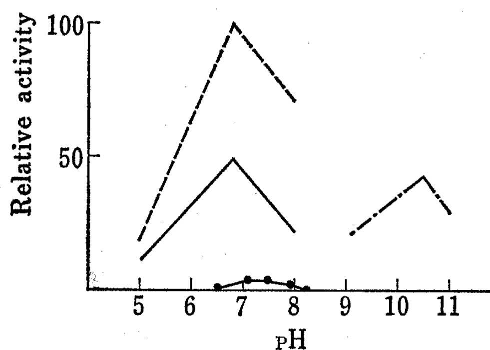

Fig. 4. Effect of pH on D-Acylase Activity

D-Acylase activity against N-benzoyl-D-phenylalanine is indicated by (------), against N-acetyl-D-phenylalanine is indicated by (------), against N-formyl-D-phenylalanine is indicated by (------), and against N-phenylacetyl-D-phenylalanine is indicated by (—●—).

Effect of pH on Activity and Stability

The effect of pH on the D-acylase activity against N-benzoyl-D-phenylalanine, N-acetyl-D-phenylalanine, N-formyl-D-phenylalanine, and N-phenyl-D-phenylalanine were measured over a pH of 5.0—11.0 with Tris-malate, Tris-HCl and carbonate buffer. As shown in Fig. 4, optimal pH against N-benzoyl-D-phenylalanine was found at the neighborhood of 10.5, against N-phenylacetyl-D-phenylalanine was at neighborhood of 7.5, against N-acetyl-D-phen-

13) P. Andrews, *Biochem. J.*, 91, 222 (1964).

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ylalanine and N-formyl-D-phenylalanine were at neighborhood of 6.8. The effect of pH on the stability of D-acylase was studied. Mixture of 1 ml of the enzyme solution and 2 ml of 0.1 M buffers of various pH range were allowed to stand at 4° for 48 hr and the enzyme activities were measured at pH 10.5. As shown in Fig. 5, the D-acylase was a relatively stable at pH 7—9 in Tris-HCl buffer but was unstable in other buffers. However, the enzyme was stabilized in the presence of glycerol.

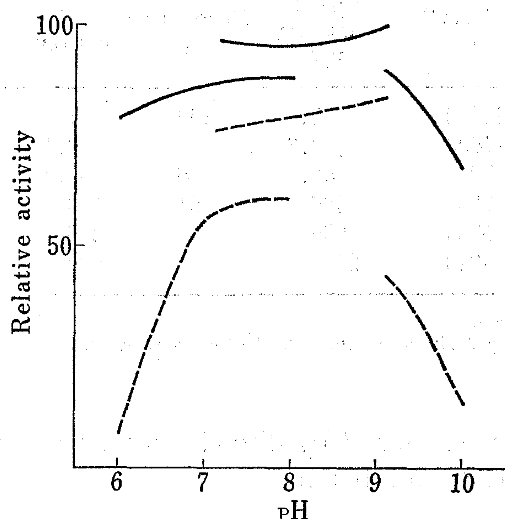

Fig. 5. Effect of pH on the Stability of D-Acylase

Mixture of 1 ml of the enzyme solution and 2 ml of 0.1 M buffer of various pH (6.0—7.0, Tris-malate; 7.0—9.0, Tris-HCl; 9.0—10.0, carbonate) were allowed to stand at 4° for 48 hr and the remaining enzyme activity were measured under the standard assay condition.
——: with 10% glycerol.
----: without glycerol.

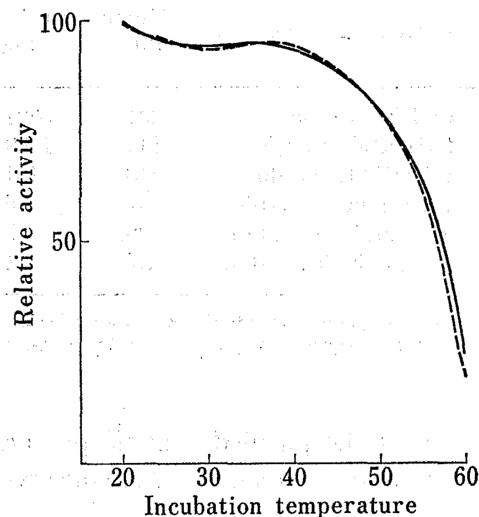

Fig. 6. Thermal Stability of the D-Acylase

The D-acylase solution were heated at indicated temperature for 5 min and the remaining activity were measured under the standard assay condition.
——: with 10% glycerol.
----: without glycerol.

Thermal Stability

The mixture of 1 ml of the enzyme solution and 1 ml of 0.01 M Tris-HCl buffer (pH 8.0) with or without glycerol was heated at the indicated temperature. After 5 min, the mixture was repidly chilled and the remaining activity was measured under the standard conditions. The result is shown in Fig. 6. The enzyme was stable up to 45° about 90% of activity remained after 5 min. But the enzyme was not stabilized in the presence of 10% glycerol.

Effect of Metal Ions, Sulfhydryl Reagents, and Other Compounds

It has been known that various L-amino acylase are specifically activated by Co^{2+} or Zn^{2+}.[14] The D-acylase activity toward N-benzoyl-D-phenylalanine was measured in the presence of metal ion, chelating agents and sulfhydryl reagents. As shown in Table III, the D-acylase was inactivated by Zn^{2+} or Hg^{2+} at 10^{-3} M, but was not affected by Co^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, or Ba^{2+} at 10^{-3} M. EDTA and *o*-phenanthroline did not inhibit the D-acylase activity at 10^{-3} M. Ascorbic acid, β-mercaptoethanol and hydrogen peroxide did not effect the activity. Diisopropylfluorophosphate (DFP) has not inhibitory effect at 10^{-3} M. L-Cysteine has a slight stimulatory effect on the D-acylase activity at 10^{-3} M, and *p*-chloromercuribenzoate (PCMB) has inhibitory effect at 10^{-3} M. In case of adenosine deaminase of calf intestinal mucosa, Ronca *et al.*[15] reported that PCMB inhibited the activity but iodoacetate did not

14) 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).
15) G. Ronca, C. Bauer, and C.A. Rossi, *Eur. J. Biochem.*, **1**, 434 (1964).

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Table III. Effects of Metal Ions, Chelating Agents, and Other Compounds on the D-Acylase Activity

Addition[a)]	Relative activity	Addition	Relative activity
None	100[b)]	$ZnCl_2$	63
Ascorbic acid	99	$CoCl_2$	97
β-Mercaptoethanol	98	$FeCl_2$	98
H_2O_2	103	$MgCl_2$	96
L-Cys	113	$CaCl_2$	99
ICH_2COOH[c)]	100	$MnCl_2$	103
DFP[c)]	100	$CuCl_2$	103
o-Phenanthroline	98	$BaCl_2$	95
EDTA	92	$HgCl_2$	4
PCMB	12		

a) Final concentration in reaction mixture of added compound was 1×10^{-3} M.
b) The activity under no addition is taken as control (100).
c) ICH_2COOH and DFP (diisopropylfluorophosphate) were incubated with the D-Acylase solution at 30° for several min, and the remaining activity was measured under standard condition.

affect the activity, and this phenomenon is explained by assuming that the sulfhydryl group may be contained in a hydrophobic region of the enzyme molecule. The D-acylase may be also explained in a manner similar to adenosine deaminase.

Substrate Specificity

In order to study the substrate specificity of D-acylase of *AAA* 6029, susceptibility of the various acyl derivatives of amino acids toward the enzyme was investigated.

1) **Optical Specificity**——As shown in Table IV, D-acylase of *AAA* 6029 could hydrolyze N-benzoyl-D-phenylalanine, N-acetyl-D-phenylalanine, N-formyl-D-phenylalanine, N-phenylacetyl-D-phenylalanine, and other N-acyl-D-amino acids. But it could not hydrolyze N-benzoyl-L-phenylalanine, N-acetyl-L-phenylalanine, N-formyl-L-phenylalanine, and N-phenyl-

Table IV. Substrate Specificity of D-Acylase of *AAA* 6029

	Benzoyl derivatives	Phenylacetyl derivatives	Acetyl derivatives	Formyl derivatives
D-Phenylalanine	100	3.0	310	115
L-Phenylalanine	0	0	0	0
D-Methionine	83	1.0	94	
L-Methionine	0	0	0	0
D-Leucine	77	0.5	70	
L-Leucine	0	0	0	
D-Alanine	40	0.4	10	
L-Alanine	0	0	0	
D-Valine	16	0.1		
L-Valine	0	0		
D-Glutamic acid	0.4	0.1		
L-Glutamic acid	0	0		
D-Aspartic acid	0.2	0.1		
L-Aspartic acid	0	0		
Glycine	0	0	0	0
D-Phenylalanine amide			0	
L-Phenylalanine amide			0	
D-Alanine amide	0			

The activity toward N-benzoyl-D-phenylalanine is 2437 units/mg and this is taken as standard (100). Digest consisting 50 μl of 0.05 M substrate, 100 μl of 0.1 M buffer and 50 μl of the enzyme solution is incubated at 30°. Activities are measured by the procedure described in "Method."

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acetyl-L-phenylalanine at a measurable rate. Thus this acylase has an optical specificity and can hydrolyze only acyl-D-amino acid, but not acyl-L-amino acid.

2) **Effect of Acyl Group**——As shown in Table IV, in the case of acyl derivatives of D-phenylalanine, N-acetyl group is the most susceptible and the susceptibility is put in order N-acetyl, N-formyl, N-benzoyl, N-phenylacetyl group. This order is not so strict, since in the case of acyl-D-alanine the order was N-benzoyl, N-acetyl, N-phenylacetyl group.

3) **Effect of Amino Acids**——As shown in Table IV, in the case of benzoyl derivatives of D-amino acids, the susceptibility for D-amino acid is D-phenylalanine>D-methionine>D-leucine>D-alanine>D-valine>D-glutamic acid. This order is the same in the case of acetyl and phenylacetyl derivatives.

As shown in Table IV, the D-acylase could not hydrolyze N-acetyl-D-phenylalanine amide, and N-benzoyl-D-alanine amide at a measurable rate. These results suggest that the D-acylase requires absolutely the presence of a free carboxyl group for effective substrate.

Ratio of D-Acylase Activities in Purification Steps

This D-acylase hydrolyzed various acyl-D-amino acids and optimal pH toward N-benzoyl-D-phenylalanine, N-acetyl-D-phenylalanine, and N-phenylacetyl-D-phenylalanine were different each other. Also, D-acylase preparation examined by disc electrophoretic analysis, had two minor components besides a main band. To clarify the question whether a few of D-acylases might be contaminated in this D-acylase preparation, the ratio of activities toward N-acyl-D-amino acids in purification steps were measured. As shown in Table V, the ratio of activities toward N-benzoyl-D-phenylalanine, N-benzoyl-D-methionine, N-acetyl-D-phenylalanine, and N-phenylacetyl-D-phenylalanine remained constant all through the purification steps. When elution pattern of these activities on DEAE cellulose chromatography and Sephadex G-100 gelfiltration were measured, the peaks of these activities overlapped each other and ratio of these activities remained constant in all fractions. These results suggested that one enzyme hydrolyzed respective acyl-D-amino acids.

α-Amino acylase are widely distributed in animals, plants, and microorganism,[16] and they act on N-acyl derivatives of L-amino acids, but not D-amino acids. Newly obtained D-acylase of *AAA* 6029 can hydrolyze N-benzoyl-D-phenylalanine, N-benzoyl-D-methionine, N-benzoyl-D-leucine, N-benzoyl-D-alanine, and N-benzoyl-D-valine. Also it hydrolyzes N-acetyl, N-formyl, and N-phenylacetyl derivatives of D-amino acid. But it can not hydrolyze N-acyl-L-amino acid such as N-benzoyl-L-phenylalanine or N-acetyl-L-phenylalanine.

TABLE V. Ratio of Activities in Purification Steps

Substrate	Step			
	Cell free extract	Ammonium sulfate fractionation	1st DEAE cellulose chromatography	Purified enzyme
N-Benzoyl-D-phenylalanine	100	100	100	100
N-Acetyl-D-phenylalanine	320	320	310	310
N-Phenylacetyl-D-phenylalanine	2.9	3.1	2.9	3.2
N-Benzoyl-D-methionine	81	82	81	83

The activity toward N-benzoyl-D-phenylalanine are 2.7 units/mg (Cell free extract), 8.3 units/mg (ammonium sulfate fractionation), 52.4 units/mg (1st DEAE cellulose chromatography), and 2437 units/mg (purified enzyme), and these are respectively taken as standard. The activity are measured by the procedure described in "Method."

16) J.P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 2, John Wiley and Sons, Inc., 1961, p. 1778; I. Chibata and T. Tosa, *Bull. Agr. Chem. Soc. Japan*, 23, 370 (1959).

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