DEACETYLATION OF PS-5, A NEW β-LACTAM COMPOUND*

III. ENZYMOLOGICAL CHARACTERIZATION OF L-AMINO ACID ACYLASE AND D-AMINO ACID ACYLASE FROM *PSEUDOMONAS* SP. 1158

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L-Amino acid acylase and D-amino acid acylase were stable below 50°C, although the D-enzyme was more thermostable than the L-enzyme at higher temperatures. At 30°C they showed the highest reaction velocity in phosphate buffer of pH 7.4. Hg^{++} and Cu^{++} severely inactivated their activity. Activation by Co^{++} was observed on L-amino acid acylase, but not on D-amino acid acylase. *p*-Chloromercuribenzoate inhibited both enzymes, whereas ethylenediamine tetraacetate was very inhibitory on L-amino acid acylase only. With N-acetyl- and N-chloroacetyl-amino acids as substrates, they were relatively stereo-specific. They acted as a peptidase on dipeptides and tripeptides. Although N-acetylglycine was attacked by the two enzymes, N-acetylglucosamine and N-acetylethanolamine were insusceptible. PS-5 was converted to NS-5 (deacetyl PS-5) by L-amino acid acylase as well as by D-amino acid acylase.

In a previous paper, the PS-5-converting activity of *Pseudomonas* sp. 1158, a facultative methanol-assimilating bacterium, was correlated with the L- and D-amino acid acylase activities.[1] These enzyme activities were found to be due to distinct proteins and were separated and partially purified.[2]

Amino acid acylase is commonly assumed to act on N-acyl amino acid only, although a very few exceptions are known (N-acetyltaurine, for example[3]). It is interesting that PS-5, a new β-lactam compound having a partial structure corresponding to the optically inactive N-acetylamino group, was deacetylated to NS-5 (deacetylated PS-5) by L-amino acid acylase from porcine kidney and D-amino acid acylase from *Streptomyces olivaceus* but not by L-amino acid acylase from *Aspergillus* sp.[1] Under these situations it seemed necessary to study the enzymological properties of L- and D-amino acid acylases from *Pseudomonas* sp. 1158, before discussing a plausible relationship between the PS-5-converting activity and the amino acid acylase activity.

The present paper describes the enzymological properties of L-amino acid acylase and D-amino acid acylase from *Pseudomonas* sp. 1158. PS-5 was deacetylated to NS-5 by the two types of acylase, whereas N-acetylglucosamine and N-acetylethanolamine were insusceptible.

Materials and Methods

Materials

The partially purified preparations of L-amino acid acylase and D-amino acid acylase from *Pseudomonas* sp. 1158 obtained in the preceding paper were employed throughout the present study[2]. L-Amino acid acylase from porcine kidney (acylase I) (catalogue No. A3010) was obtained from Sigma Chemical Co., St. Louis, MO., U.S.A. D-Amino acid acylase from *Streptomyces olivaceus* (a crude preparation from sonicate by ammonium sulfate fractionation) was a gift of SUGIE and SUZUKI[4]. PS-5 sodium salt was prepared and checked for purity in our laboratories as described in a previous paper[1].

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N-Acetylglycine, N-acetylglucosamine and N-acetylethanolamine were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. As N-acetylethanolamine was found to contain some ninhydrin-positive impurities, it was purified as follows: 15 ml of N-acetylethanolamine was dissolved in 135 ml of distilled water and passed through a column (75 ml in capacity) of a 1: 1 mixture of Amberlite IR-120B (H^+) and Amberlite IR-410 (OH^-). After water was removed by evaporation, the residue was dried with molecular sieves 4A. This preparation of N-acetylethanolamine was proved to be ninhydrin-negative on thin-layer chromatography. Its infra-red spectrum was identical with that in the literature.

Other substrate compounds listed in the present paper were obtained from Sigma Chemical Co. and were used without preliminary purification.

Other reagents were commercially available products of analytical grade.

Enzyme assay procedures

The standard assay conditions for L-amino acid acylase and D-amino acid acylase were described in a previous paper[1].

Thermostability of the enzymes was determined by measuring the remaining acylase activity after pre-incubation for 15 minutes at the indicated temperatures.

The optimal reaction temperature was examined by comparing the reaction velocities when the standard assay mixtures were incubated at the indicated temperatures.

For the pH stability test, the enzymes were kept at 4°C for 20 hours in 20 mM (final concentration) of sodium citrate buffer (pH 4~5), potassium phosphate buffer (pH 6~8), Tris-HCl buffer (pH 7.5~9), sodium borate buffer (pH 8~9) and Veronal buffer (pH 8.5~9.5), and then the enzyme solutions were diluted two-fold with 0.25 M potassium phosphate buffer of pH 7.4 before amino acid acylase assay.

The optimal reaction pH for deacylation was determined in sodium citrate buffer (pH 4~5), potassium phosphate buffer (pH 6~8) and Veronal buffer (pH 8.5~9.5).

The effects of metal ions on the acylases were studied under the standard assay conditions by replacing $CoCl_2$ by the indicated metal ions.

The effects of inhibitors on L- and D-amino acid acylases were examined by pre-incubating the enzymes with 1 mM of the indicated compounds for 15 minutes at 30°C before assay for the residual enzyme activity.

For the qualitative assay of the peptidase activity, the reaction period of time was prolonged to 60 minutes and reaction products were analysed by silica gel thin-layer chromatography, as described in a previous paper[1].

Disc gel electrophoresis for location of the peptidase activity

Under the same experimental conditions as specified for location of the amino acid acylase activity in the preceding paper[2], the two enzyme preparations were submitted to polyacrylamide disc gel electrophoresis. The peptidase activity in 1-mm sections of the gel was assayed with L-leucyl-L-leucine for L-amino acid acylase and with L-leucyl-D-leucine for D-amino acid acylase.

Deacetylation of PS-5 to NS-5

The deacetylation conditions were same as detailed in a previous paper[1]. Conversion of PS-5 to NS-5 was semi-quantitatively estimated by means of high voltage paper electrophoresis followed by bioautography with *Staphylococcus aureus* FDA 209P[1].

Results

1. Thermostability

Fig. 1 shows the thermostability of L-amino acid acylase and D-amino acid acylase relative to the control activity observed at 5°C.

Both acylases were fairly stable at a temperature below 50°C. At higher temperatures, L-amino acid acylase rapidly lost activity to reach zero at 70°C, whereas D-amino acid acylase was thermostable, showing 30% of the control activity even at the highest temperature tested (77°C).

Fig. 1. Thermostability of L- and D-amino acid acylases from *Pseudomonas* sp. 1158.
For the reaction conditions and assay method see the text.

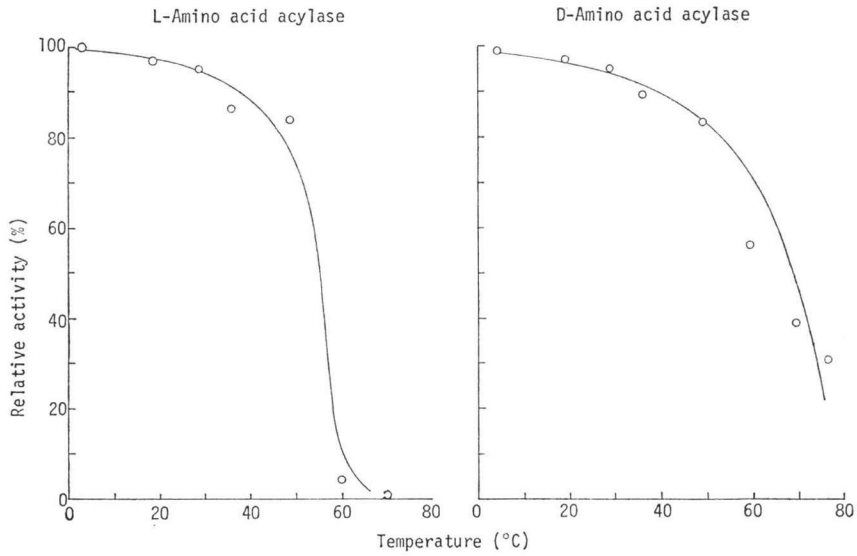
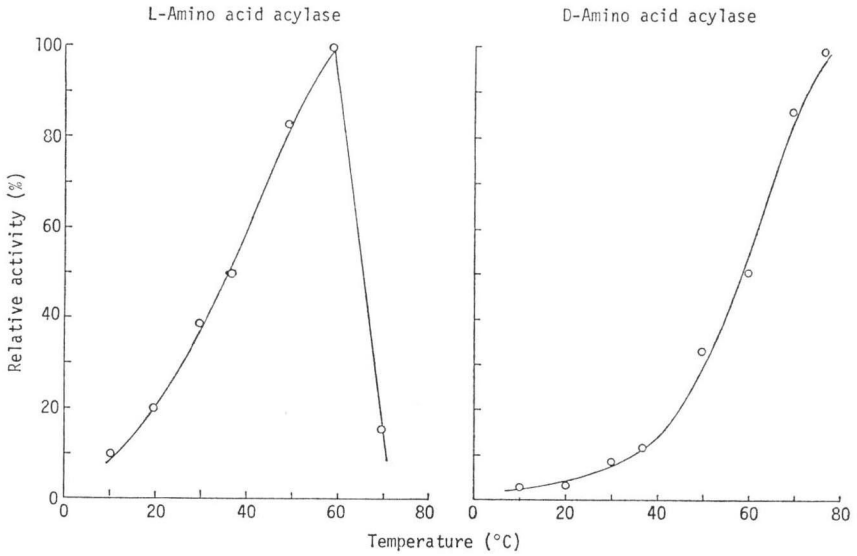


Fig. 2. Optimal reaction temperature for L- and D-amino acid acylases from *Pseudomonas* sp. 1158.
For the reaction conditions and assay method see the text.



2. Optimal Reaction Temperature

The effect of the incubation temperature on the reaction velocity is presented in Fig. 2, referring as 100 to the highest reaction velocity observed.

As is the case in other known L-amino acid acylases,[5,6] the reaction velocity of the L-acylase of *Pseudomonas* sp. 1158 rose with the incubation temperature upto 60°C which was very close to the temperature for complete inactivation (*cf.* Fig. 1). D-Amino acid acylase, as was expected, was found to be

Fig. 3. pH Stability of L- and D-amino acid acylases from *Pseudomonas* sp. 1158.
For the reaction conditions and assay method see the text. (□) sodium citrate buffer, (○) potassium phosphate buffer, (●) Tris-HCl buffer, (■) sodium borate buffer, (△) Veronal buffer.

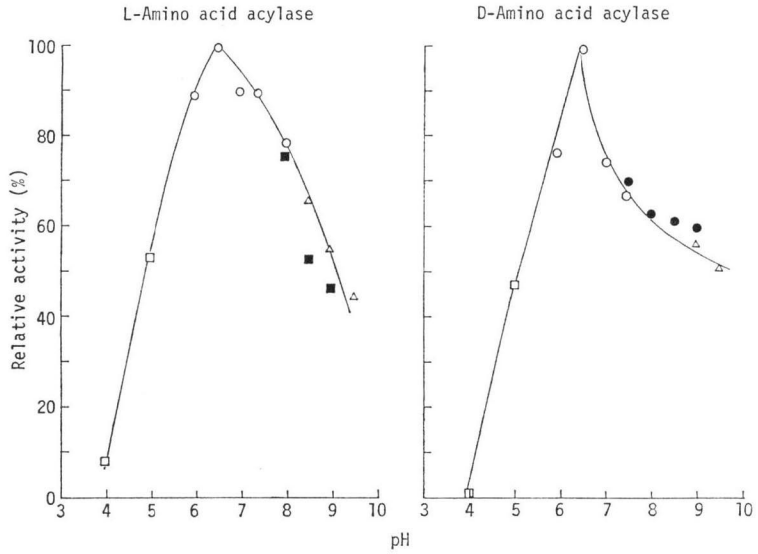
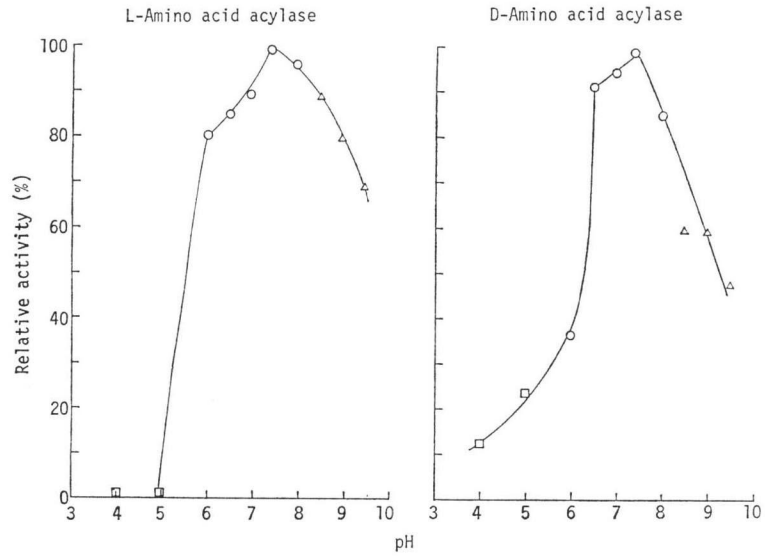


Fig. 4. Optimal reaction pH for L- and D-amino acid acylases from *Pseudomonas* sp. 1158.
For the reaction conditions and assay method see the text. (□) sodium citrate buffer, (○) potassium phosphate buffer, (△) Veronal buffer.



most active at the highest temperature tested (77°C). It is uncertain whether the D-amino acid acylase preparation contained some thermostabilizing factor or not.

3. pH Stability

The enzymes were most stable at pH 6.5 and were inactivated more severely at acidic pH's than at

alkaline pH's (Fig. 3).

4. Optimal Reaction pH

When N-chloroacetyl-L-valine and N-chloroacetyl-D-valine were employed as substrates for L-amino acid acylase and D-amino acid acylase respectively, the optimal pH value for deacetylation was found to be 7.4 in potassium phosphate buffer for both acylases (Fig. 4).

Table 1. Effects of divalent metal ions on L- and D-amino acid acylases from *Pseudomonas* sp. 1158.

Metal ion	Concn. (mM)	Relative activity (%)	
		L-Amino acid acylase	D-Amino acid acylase
Hg^{++}	0.01	90.5	28.5
	0.1	4.6	17.8
Cu^{++}	0.1	41.7	28.4
Mn^{++}	1.0	70.2	98.7
Zn^{++}	1.0	84.5	83.6
Cd^{++}	1.0	51.4	96.1
Sn^{++}	1.0	53.2	89.6
Pb^{++}	1.0	92.3	88.2
Mg^{++}	1.0	77.2	91.3
Fe^{++}	1.0	75.4	90.6
Ni^{++}	1.0	107.7	97.6
Co^{++}	0.01	93.1	
	0.05	104.2	
	0.1	110.5	
	0.5	153.2	
	1.0	160.5	100.1
	10.0	146.8	
None		100.0	100.0

Table 2. Effects of inhibitors on L- and D-amino acid acylases of *Pseudomonas* sp. 1158.

Inhibitor	Concn. (mM)	Relative activity (%)	
		L-Amino acid acylase	D-Amino acid acylase
N-Ethylmaleimide	1	108.5	78.1
Monoiodoacetate	1	97.1	72.9
NaN_3	1	100.2	103.3
p-CMB	1	17.5	25.3
EDTA	1	7.3	88.1
Sodium citrate	1	83.6	90.7
KCN	1	98.3	88.8
N-ClAc-D-Val	5	98.4	
N-ClAc-L-Val	5		98.1
None		100.0	100.0

p-CMB=*p*-Chloromercuribenzoate
EDTA=Ethylenediamine tetraacetate

5. Effects of Divalent Metal Ions

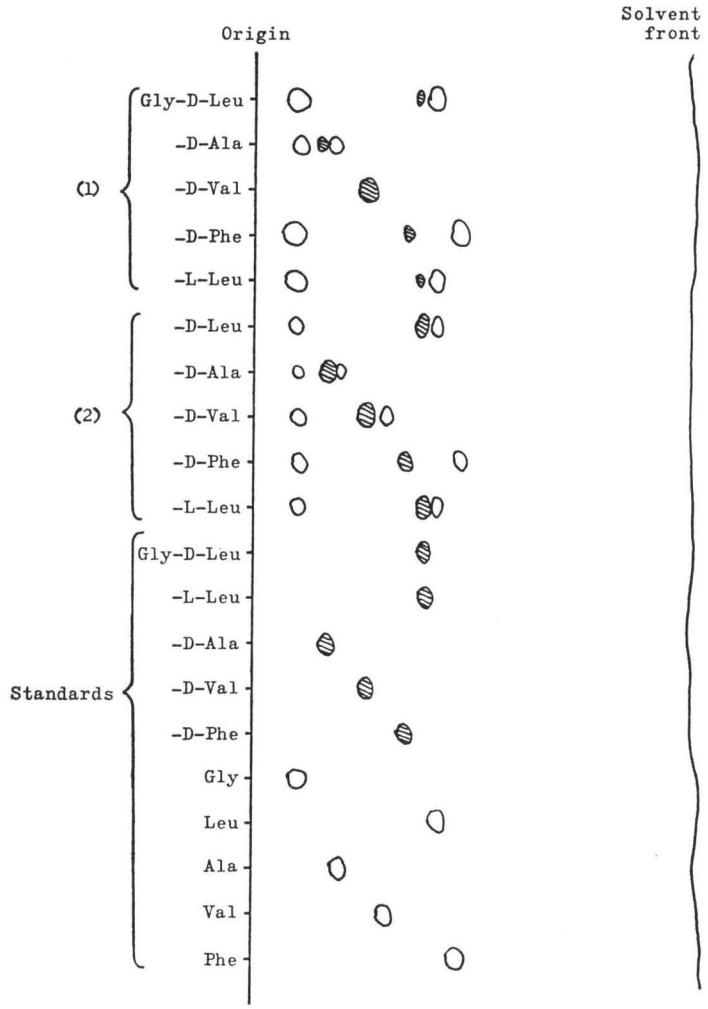
It is well acknowledged that L-amino acid acylase is substantially activated by Co^{++}.[7] With N-chloroacetylvalines as substrates, the effects of various metal ions on L- and D-amino acid acylases were examined by replacing Co^{++} by the indicated ions in the standard assay mixtures. The results are summarized in Table 1.

Hg^{++} and Cu^{++} ions strongly inhibited L- and D-amino acid acylases. In general, L-amino acid acylase was more sensitive to divalent metal

Table 3. Substrate profiles of L- and D-amino acid acylases from *Pseudomonas* sp. 1158.

Substrate	Relative activity (%)	
	L-Amino acid acylase	D-Amino acid acylase
N-Ac-L-Met	100.0	10.3
D-Met	0	100.0
L-Val	25.3	8.9
D-Val	10.0	17.9
L-Ala	134.6	0
D-Ala	0	96.2
L-Try	13.3	0
D-Try	0	40.2
L-Phe	24.6	0
D-Phe	0	38.9
L-Leu	113.0	0
D-Leu	0	34.9
L-Asn	9.1	0
D-Asn	0	33.3
L-Glu	24.0	
N-ClAc-L-Val	1105.4	0
D-Val	11.3	178.3
L-Leu	2060.8	
L-Phe	1650.0	
N-Formyl-L-Met	863.5	
L-Leu	791.9	
N-α-Ac-L-Lys	0	
N-Ac-Gly	168.8	30.1

Fig. 5. Peptidase activity on glycyldipeptides.
For the reaction conditions and assay method see the text. (1) L-amino acid acylase from *Pseudomonas* sp. 1158, (2) D-amino acid acylase from *Pseudomonas* sp. 1158. Hatched spots show substrate.



ions than D-amino acid acylase, and Cd^{++} and Sn^{++}, for example, inhibited the L-enzyme by 50% at 1 mM, while their effects on the D-enzyme were slight.

Activation by Co^{++} was concentration-dependent for L-amino acid acylase. D-Amino acid acylase, on the other hand, was least influenced by Co^{++}, indicating a possibility that the D-enzyme might tightly bind with the Co^{++} ion, or that it might require no Co^{++} ion for activity.

6. Effects of Inhibitors

Table 2 shows the effects of various inhibitors on the two types of amino acid acylase from *Pseudomonas* sp. 1158.

p-Chloromercuribenzoate, an SH inhibitor, strongly inhibited L- as well as D-amino acid acylase. As is consistent with the difference in Co^{++} activation, ethylenediamine tetraacetate had no clear influence on D-amino acid acylase, whereas L-amino acid acylase was markedly inhibited. The optical isomer of substrate seemed not to be inhibitory at 5 mM. N-Ethylmaleimide and monoiodoacetate were

found to be more inhibitory on D-amino acid acylase than on L-amino acid acylase, although their effects were less significant.

7. Substrate Specificity as Amino Acid Acylase

Relative rates of deacylation of L-amino acid acylase and D-amino acid acylase from *Pseudomonas* sp. 1158 are collectively presented in Table 3, referring as 100 to their activities on N-acetyl-L- and D-methionines respectively.

Except N-acetylvalines and N-acetylmethionines, the substrate compounds tested indicated the strict optical specificity in relation with susceptibility. As the chemical purity of N-acetyl-L- and D-valines employed in the present work was not checked, and as the two enzyme preparations were accompanied by several minor proteins[2], it is uncertain whether the loose substrate specificity on N-acetyl-valines was intrinsic in the amino acid acylase activities. Optically inactive N-acetylglycine was attacked by both enzymes. This is also reported for D-aminoacylase from *Streptomyces olivaceus*[4].

Ease of deacylation of N-acyl-L-valines by L-amino acid acylase was in the decreasing order of N-chloroacetyl-L-valine, N-formyl-L-valine and N-acetyl-L-valine. L-Amino acid acylase from porcine kidney[7] and D-amino acid acylase from *Pseudomonas* sp. AAA6029[8] are reported to show the following decreasing order of hydrolysis: N-chloroacetylamino acids, N-acetylamino acids, N-formylamino acids.

8. Peptidase Activity

Some N-glycyldipeptides can be a substrate for L-amino acid acylase[7,9]. In addition, there are some carboxypeptidases that deacylate N-acylamino acids[10]. Thus the peptidase activity of the two acylase preparations was qualitatively checked by thin-layer chromatography. Fig. 5 presents the results of assay with five N-glycyldipeptides.

It is strange to note that, except in the combination of L-amino acid acylase with N-glycyl-D-valine, the two acylase preparations of *Pseudomonas* sp. 1158 seemed to act nonspecifically on the dipeptides tested irrespective of the optical property of the C-terminal amino acid.

As the four types of leucylleucine were commercially available, they were tested as possible substrates (Fig. 6).

The assay results shown in Fig. 6 can hardly be correlated with their substrate specificities as L- and D-amino acid acylases (Table 3).

Since the two acylase preparations used in this paper were found to be non-homogeneous on polyacrylamide disc gel electrophoresis (Fig. 2 of the preceding paper), it seemed necessary to examine the possibility that the peptidase activity might be present in protein bands other than the amino acid acylase activity. Thus, with L-leucyl-L-leucine and L-leucyl-D-leucine as substrates, the peptidase activities were located in the disc gel electrophoretograms of the L- and D-amino acid acylase preparations respectively. In both electrophoretograms, the peptidase activity was proved to correspond with the amino acid acylase activity (Figure not shown). Moreover they contained no protein other than the amino acid acylase peak that acted on the test substrate.

When glycylglycyl-L- and D-leucines were incubated with the two acylases of *Pseudomonas* sp. 1158, the thin-layer chromatographic analysis gave the results shown in Fig. 7.

As is observed with leucylleucines, the seemingly strict substrate specificity for N-acylamino acids could not be recorded. A noticeable difference between the L-amino acid acylase and the D-amino acid acylase was observed in the formation of glycylleucine; that is, glycylleucine was not produced by the L-acylase, whereas the D-acylase clearly formed it from the two tripeptides. Accordingly it is likely that

Fig. 6. Peptidase activity on leucylleucines.

For the reaction conditions and assay method see the text. (1) L-amino acid acylase from *Pseudomonas* sp. 1158, (2) D-amino acid acylase from *Pseudomonas* sp. 1158. Hatched spots show substrate.

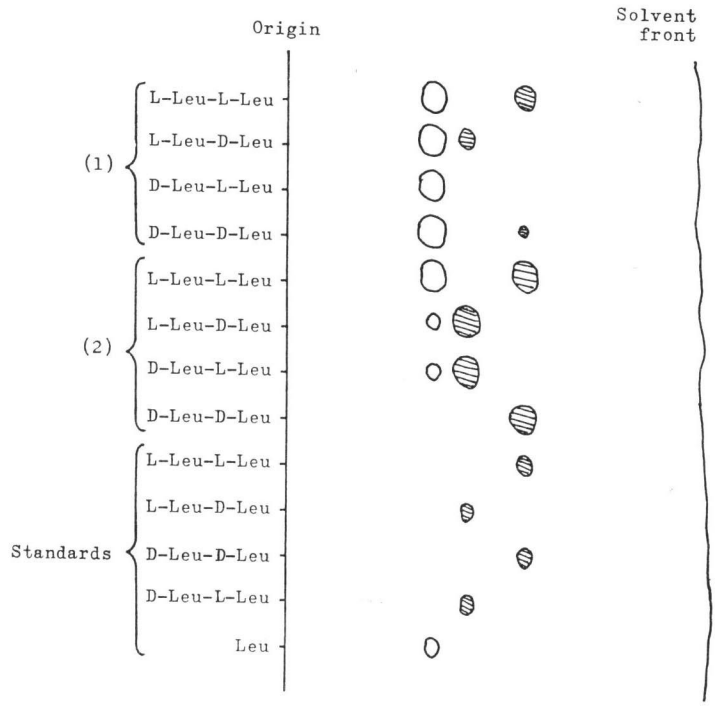
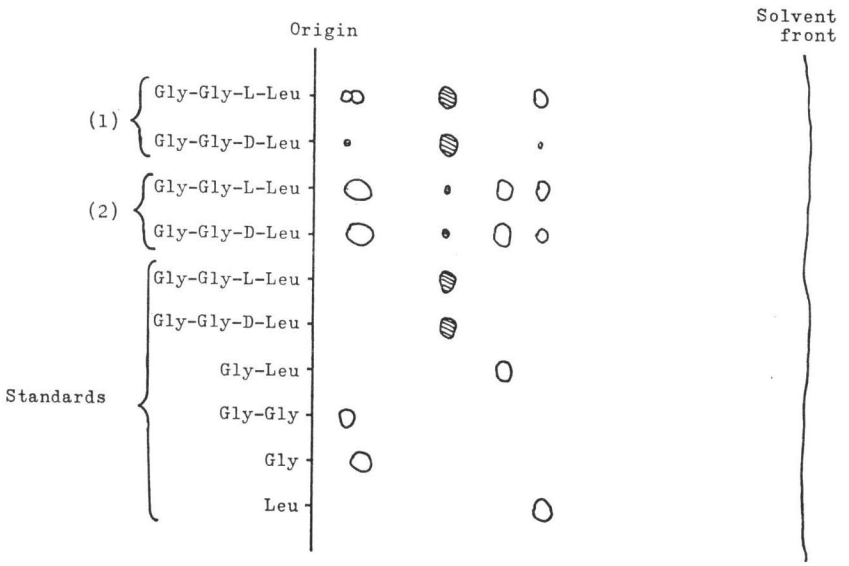


Fig. 7. Peptidase activity on glycylglycylleucines.

For the reaction conditions and assay method see the text. (1) L-amino acid acylase from *Pseudomonas* sp. 1158, (2) D-amino acid acylase from *Pseudomonas* sp. 1158. Hatched spots show substrate.



the L-amino acid acylase split the peptides from the C-terminal, whereas the D-enzyme did so from the N-terminal.

9. Action on N-Methylamino Acids, N-Acetylglucosamine and N-Acetylethanolamine

Under the standard assay conditions, sarcosine (=N-methylglycine) and N-methyl-L-leucine were resistant to the two types of amino acid acylase from *Pseudomonas* sp. 1158. The enzymes could not attack either N-acetylglucosamine or N-acetylethanolamine at all.

10. Activity of PS-5-Conversion to NS-5

As a quantitative assay method for PS-5 and NS-5 was not available, the relative PS-5-converting activities of the relevant amino acid acylases could not be compared kinetically. In Fig. 8 the bioautographic assay results with *Staphylococcus aureus* FDA 209P are presented.

In addition to L-amino acid acylase from porcine kidney and D-amino acid acylase from *Streptomyces olivaceus*[1], both L-amino acid acylase and D-amino acid acylase from *Pseudomonas* sp. 1158 deacetylated PS-5 to give NS-5. Since PS-5 is neither α- nor β-amino acid, it is very strange that the deacetylation of PS-5 to NS-5 is catalysed by L- and D-amino acid acylases. Although no plausible explanation is at present available on the relationship of the proper acylase activity with the PS-5-converting activity, the sulfur atom in the PS-5 molecule seems to play an important role in the determination of the substrate affinity with acylases. FUJIMOTO *et al.*[3] reported that N-acetyl-β-alanine deacetylase from hog kidney deacetylated N-acetyltaurine, but not N-acetyl-L-alanine, N-acetylglycylglycine or N-acetylglycyl-β-alanine. Moreover, when the sulfur atom of PS-5 was oxidized to give PS-5 sulfoxide, 3-(2-acetamidoethyl)sulfynyl-6-ethyl-7-oxo-1-azabicyclo [3. 2. 0] hept-2-ene-carboxylate, it became resistant not only to the L- and D-amino acid acylases of *Pseudomonas* sp. 1158 but also to the acylases from porcine kidney and *Streptomyces olivaceus*.

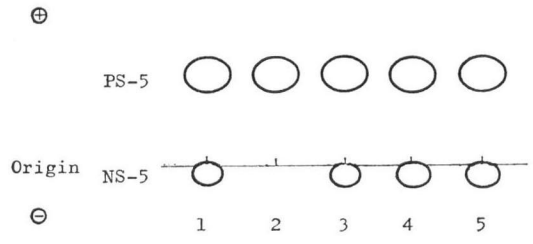


Fig. 8. Conversion of PS-5 to NS-5 by various amino acid acylases.

For the reaction conditions and assay method see the text.

(1) L-Amino acid acylase from porcine kidney, (2) L-Amino acid acylase from *Aspergillus* sp., (3) D-Amino acid acylase from *Streptomyces olivaceus*, (4) L-Amino acid acylase from *Pseudomonas* sp. 1158, (5) D-Amino acid acylase from *Pseudomonas* sp. 1158.

Discussion

As enzyme which deacylates various N-acyl-L-amino acids, acylase I (EC 3.5.1.14; L-amino acid acylase in hog kidney)[7], acylase II (EC 3.5.1.15; N-acetylaspartic acid acylase in hog kidney)[12], acylase III (N-acyl-L-aromatic amino acid deacylase in hog kidney)[13], N-acetyl-β-alanine deacetylase (EC 3.5.1.12; hog kidney)[3] and L-amino acid acylases from plants and microorganisms[6,9] are known in the literature. D-Amino acid acylases, on the other hand, are found in *Streptomyces*[4] and *Pseudomonas*.[8] DL-Amino acid acylase from *Pseudomonas* which does not act on N-acetylamino acids is described by KAMEDA *et al.*[14]

In comparison with the known types of amino acid acylases, L-amino acid acylase and D-amino acid acylase from *Pseudomonas* sp. 1158 are very peculiar in their substrate profiles. They seemingly revealed a relatively strict substrate specificity for N-acetylamino acids (Table 3), but their peptidase activities were

noticeable and least stereo-specific on dipeptides and tripeptides (Figs. 5, 6 and 7).

The olivanate family of β-lactam compounds including PS-5 are neither α- nor β-amino acid and thus the susceptibility of PS-5 to L- and D-amino acid acylases should be considered illegitimate. Although only limited information is available on the relationship of the proper amino acid acylase activity with the PS-5-converting activity, the peptidase activity of amino acid acylase seems to be involved in the conversion of PS-5 to NS-5.

The Merck research group has recently disclosed in the Japan Kokai Patent specifications that penicillin acylase from *Escherichia coli* acylated and deacylated the thienamycin group of β-lactam compounds[11,15] and that N-acetylethanolamine amidohydrolase from *Protaminobacter ruber* deacylated thienamycin-related compounds[16,17]. In our study, L-amino acid acylase and D-amino acid acylase from *Pseudomonas* sp. 1158 did not show any activity on N-acetylethanolamine (see the text). In a previous paper[1], two strains of *Protaminobacter ruber*, both of which produced L-amino acid acylase only, were found to be weakly active on PS-5.

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