STUDIES ON THE BIOSYNTHESIS OF CARBAPENEM ANTIBIOTICS

III. ENZYMOLOGICAL CHARACTERIZATION OF THE L-AMINO ACID ACYLASE ACTIVITY OF A933 ACYLASE

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A933 acylase, which is involved in exchange of the pantothenyl substituent of OA-6129 carbapenems with acetyl CoA, was characterized as an L-amino acid acylase with a molecular weight of 100,000 (±8,000) and a pI value of 5.1. The highest L-amino acid acylase activity of A933 acylase was observed at 37°C and pH 7~7.5 for *N*-chloroacetyl-L-phenylalanine. Unlike other amino acid acylases, A933 acylase was severely inhibited by cobalt ions and *p*-chloromercuribenzoate. The acylase also showed peptidase activity with some di- and tripeptides. A protein fraction with A933 L-amino acid acylase activity from blocked mutant 1501 lacked OA-6129A-depantothenylating activity.

A933 acylase is a multi-functional key enzyme in the biosynthesis of carbapenems which catalyzes the conversion of OA-6129A, B_1, B_2 and C to PS-5, epithienamycins A and C and MM 17880, respectively, in the presence of acetyl CoA (Fig. 1).[1,2] In view of the previous finding that PS-5 is deacetylated by certain L- and D-amino acid acylases, which also attack dipeptides and tripeptides,[3] a detailed characterization of the L-amino acid acylase activity of A933 acylase was needed to comparatively evaluate the reaction mechanisms of L- and D-amino acid acylases, penicillin acylase and A933 acylase and to elucidate the biosynthesis and enzyme susceptibility of β-lactams. Like penicillins and cephalosporins, carbapenems are assumed to be synthesized from amino acids and are recognized *in vivo* as dipeptides by dipeptidase or dehydropeptidase I.[4]

In the biosynthesis of gramicidins and tyrocidines, the pantetheine moiety in the multi-enzyme unit functions as the carrier arm for peptide elongation.[5] Pantetheine has essentially the same role in the carbon chain elongation of fatty acids.[6] In the biosynthesis of carbapenems, however, at least one mol of pantetheine appears to be consumed in the production of one mol of the antibiotic, judging from the structure of the OA-6129 group of carbapenems.

An enzyme called pantetheinase is reported to be involved in the metabolism of CoA, although its amino acid acylase activity, and further its possible identity with acylase I or L-amino acid acylase, have not yet been examined.[7]

The present paper describes the physico-chemical and enzymological properties of A933 acylase. Contrary to our expectation, *Streptomyces fulvoviridis* A933 1501, a blocked mutant producing the OA-6129 group of carbapenems, retained the L-amino acid acylase activity, but no longer OA-6129A-depantothenylating activity.

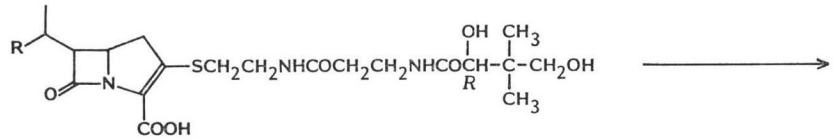
Materials and Methods

Materials

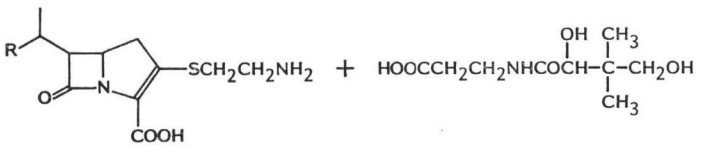
A partially purified preparation of A933 acylase was isolated from *Streptomyces fulvoviridis*

Fig. 1. Reactions catalyzed by A933 acylase.[2)]

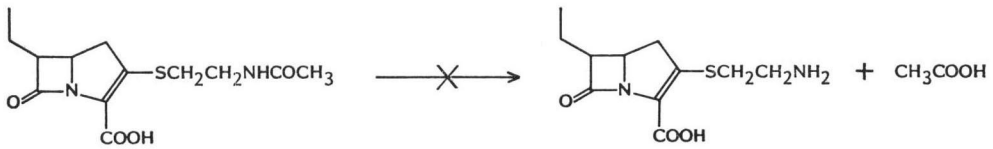
(1) Depantothenylation of OA-6129 carbapenems.



OA-6129A R = H
OA-6129B$_1$ R = OH
OA-6129B$_2$ R = OH
OA-6129C R = OSO_3H

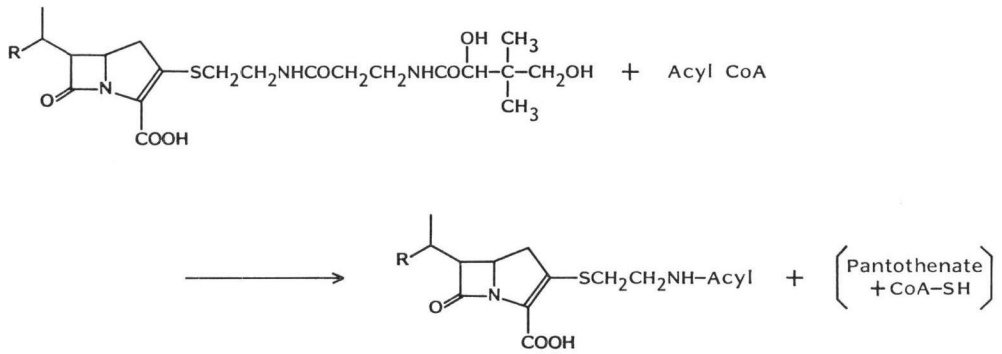


(2) Deacylation of acyl-L-amino acids without deacetylation of PS-5.

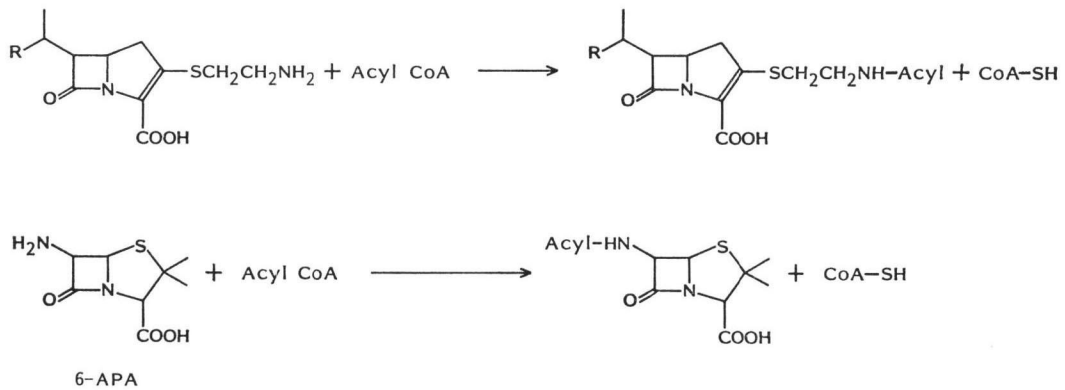


PS-5 NS-5

(3) Acyl exchange of OA-6129 carbapenems with acyl CoA.



(4) Acylation of deacylated carbapenems and 6-APA.



6-APA

A933-17M9 as described in a previous paper.[2] *N*-Acyl amino acids, peptides, 1,4-piperazinediethanesulfonic acid (PIPES), acylase I (L-amino acid acylase), L-amino acid oxidase, peroxidase and *o*-dianisidine were purchased from Sigma Chemical Co.; and a protein marker kit consisting of cytochrome *c* (horse heart), chymotrypsinogen A (bovine pancreas), albumins (egg white and bovine serum), aldolase (rabbit muscle) and catalase (beef liver) was obtained from Boehringer Mannheim Co., Ltd. Bio-Lytes (carrier ampholytes 4/6 and 3/10) and an analytical electrofocusing apparatus were from Bio-Rad Laboratories. Other reagents were commercially available products of analytical or higher grade.

Enzyme Assay Procedures

For the standard assay of L-amino acid acylase activity, *N*-chloroacetyl-L-phenylalanine was employed as the substrate. The assay substrate was dissolved in distilled water and adjusted with 2 N NaOH to a pH in the range of 6.5~7.5. A reaction mixture contained, in a final volume of 50 μl, 1 μmol of substrate (final concentration 20 mM), 2 μmol of PIPES (40 mM), pH 7.3, and enzyme. After incubation at 37°C for 15 minutes, the reaction mixture was instantly cooled in dry ice - acetone and mixed with 50 μl of 50% acetic acid for termination of the enzyme reaction. The amount of the amino acid released was quantitatively analyzed with ninhydrin by the method of YEMM and COCKING[8] or with an automatic amino acid analyzer (Hitachi 835).

For thermostability tests, the A933 acylase preparation was pre-incubated for 15 minutes at the indicated temperatures; and the remaining L-amino acid acylase activity was measured under the standard assay conditions. The temperature-dependence of the acylase was examined by comparing the rates of deacylation at the indicated temperatures.

The pH stability test was carried out by preincubating the enzyme at 5°C for 15 hours in 40 mM (final concentration) acetate (pH 5~6), PIPES (pH 6~8) or Veronal (pH 8.6~9.4) buffer, followed by a standard assay of the remaining activity. The pH dependence of the acylase was also measured in the above three buffer systems.

The effect of metal ions on the L-amino acid acylase activity of A933 acylase was studied using the standard assay conditions with supplements of metal ions at the indicated concentrations.

The influences of inhibitors on the acylase were measured after preincubation of the enzyme with 1 mM (final concentration) of the indicated compounds for 15 hours at 5°C or for 15 minutes at 37°C.

Isoelectric Focusing

A thin-layer of polyacrylamide gel containing 1.6% Bio-Lyte 3/10 and 0.4% Bio-Lyte 4/6 was prepared according to the technical information from Bio-Rad Laboratories. After electrophoresis at 0~4°C for 4 hours (at a constant power of 5 watts), the pH gradient established on the gel electrophoretogram was recorded with a micro-electrode. For location of the L-amino acid acylase activity, 0.6% agarose solution containing 10 mM (final concentration) *N*-chloroacetyl-L-phenylalanine (pH 7.0), 0.5 mM $MnCl_2$, 0.05% L-amino acid oxidase (snake venom), 0.05% peroxidase (horse radish), 0.08% *o*-dianisidine and 200 mM potassium phosphate buffer, pH 7.4, was overlaid at 45°C on the electrophoretogram, and incubated at 37°C for 60 minutes. The treated electrophoretogram was developed in 5% acetic acid. The L-amino acid acylase activity was visualized with reduced *o*-dianisidine as a brownish orange band as a result of the following reaction sequence:[9]

N-Acyl-L-amino acid + H_2O ⋯⋯→ L-amino acid + acyl-OH

L-Amino acid + O_2 + H_2O ⋯⋯→ α-keto acid + NH_3 + H_2O_2

H_2O_2 + *o*-dianisidine·H_2 ⋯⋯→ $2H_2O$ + *o*-dianisidine·dye

Disc Gel Electrophoresis

Disc gel electrophoresis was run by the method of DAVIS[10] except that the spacer gel was replaced by 40% sucrose containing the enzyme which was applied directly on the coarse gel. Electrophoretic conditions were as follows: pH 8.3, current 2 mA/disc (5 mm ID × 50 mm), 30 minutes at 4°C. After electrophoresis using bromophenol blue as a marker, the disc was longitudinally sliced into halves. One half of the disc gel was stained for protein bands with Coomassie Brilliant Blue R. To locate L-amino acid acylase activity, the other half of the disc gel was sliced transversely into 1-mm sections.

Each section was macerated in a reaction mixture containing 50 μl of 0.2 M phosphate buffer, pH 7.1, and 50 μl of 100 mM *N*-chloroacetyl-L-phenylalanine; and incubated at 37°C for 60 minutes. The resulting reaction mixture was treated for 5 minutes in boiling water; 2 μl of the supernatant solution was applied to a silica gel TLC plate for amino acid analysis.

Estimation of the Molecular Weight

The acylase was subjected to gel filtration on Sephadex G-150, as described by ANDREWS.[11] The void volume of the Sephadex G-150 column (19×560 mm) was determined with Blue Dextran 2000, and the elution volumes of the marker proteins were spectrophotometrically read at 280 nm.

Quantitation of Protein

The protein concentration was spectrophotometrically measured by the method of KALB, Jr., and BERNLOHR.[12]

Results and Discussion

Classification of Amidohydrolases by Their Action on PS-5 and OA-6129A

As reported in a previous paper,[2] A933 acylase catalyzes depantothenylation of OA-6129A, but not deacetylation of PS-5. On this basis, the acylase can also be classified as a "pantetheinase".[7,13] Furthermore A933 acylase exhibits a so-called penicillin acylase and an L-amino acid acylase activity.[2] In addition to deacylation of acyl amino acids, acylase I from hog kidney[14] and L- and D-amino acid acylases from *Pseudomonas* sp. 1158[3] not only depantothenylate OA-6129A but also deacetylate PS-5, whereas Amano acylase 15000 from *Aspergillus* sp.[15] has neither of these activities. The pseudomonal acylases also hydrolyze dipeptides and tripeptides seemingly without stereo-specific substrate specificity.[3]

The above-described findings allow us to predict that enzymes such as pantetheinase, penicillin acylase, carboxypeptidase[16] and probably dipeptidase,[17] which are all covered by the general term amidohydrolase, may have some types of amino acid acylase activity. Their actions on PS-5 and OA-6129A, together with their stereo-specificity as amino acid acylases, indicate that they can be classified into the three types shown in Table 1.

A933 acylase is specific for the biosynthesis of carbapenem antibiotics and is classified as type III. The other acylases or enzymes which show acylase activity are classified in type I or type II.

Characterization of the L-Amino Acid Acylase Activity of A933 Acylase

Physico-chemical Properties

The molecular weight of A933 acylase was estimated to be 100,000 (±8,000) from the elution pattern of the enzyme and protein markers on Sephadex G-150 (Fig. 2).

Polyacrylamide gel electrofocusing revealed that the isoelectric point of the acylase was 5.1. It showed a relative mobility (R_m) of 0.57 against bromophenol blue in disc gel electrophoresis. Although the molecular weight and isoelectric point are known for various acylases (porcine kidney

Table 1. Classification of various acylases based on activities toward PS-5 and OA-6129A.

Type	Deacetylation of PS-5	Depantothenylation of OA-6129A	Examples
I	+	+	Hog kidney acylase (acylase I) *Pseudomonas* L- and D-amino acid acylases
II	−	−	*Aspergillus* L-amino acid acylase
III	−	+	A933 acylase

Fig. 2. Molecular weight estimation of A933 acylase.

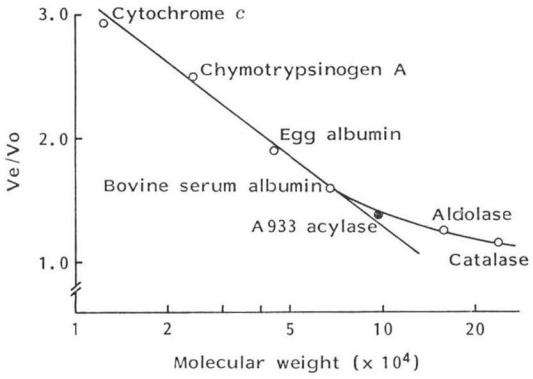


Fig. 3. Thermostability of A933 acylase.

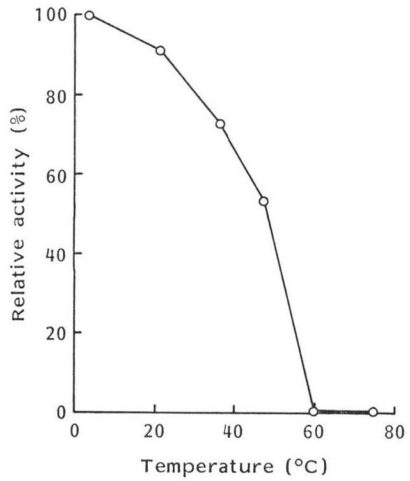
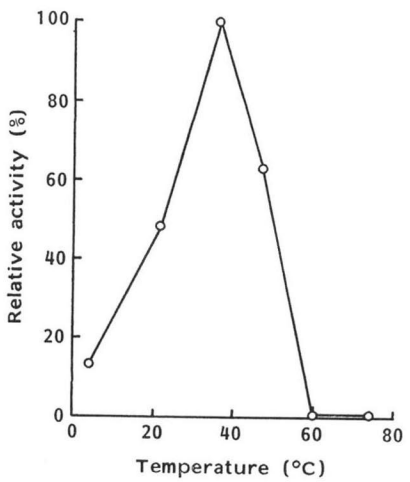


Fig. 4. Temperature-dependence of A933 acylase.



L-amino acid acylase MW=119,000;[14] *Pseudomonas* sp. 1158 L-amino acid acylase MW=75,000, pI=4.95;[18] D-amino acid acylase MW=100,000, pI=5.45;[18] *Streptomyces olivaceus* D-amino acid acylase MW=45,000[19]), including pantetheinase (MW=55,000, pI=4.8[13]) and penicillin acylases (*e.g.*, *Escherichia coli* MW=71,000, pI=6.8[20]), relative mobility data which are useful for quick characterization on gel electrophoresis have not yet been reported.

Fig. 5. pH stability of A933 acylase.
△: Acetate buffer, ○: PIPES buffer, □: Veronal buffer.

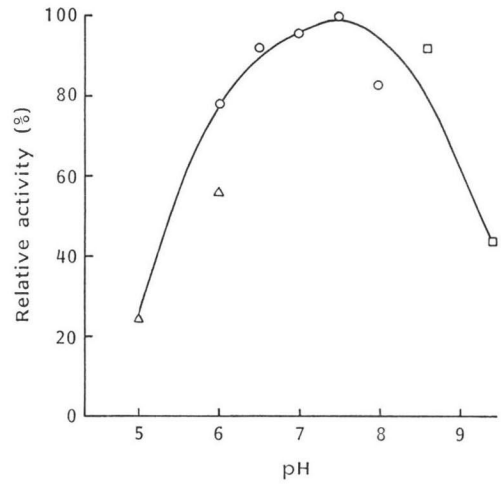
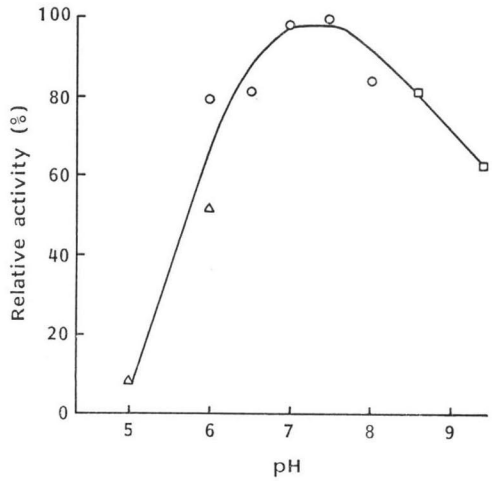


Fig. 6. pH-dependence of A933 acylase.
△: Acetate buffer, ○: PIPES buffer, □: Veronal buffer.



Thermostability and Temperature-dependence

Fig. 3 illustrates the thermostability of A933 acylase relative to a control sample stored at 4°C. When incubated at pH 7.3 for 15 minutes, the A933 acylase preparation lost about a half of its L-amino acid acylase activity at 47°C, and all at 60°C. Compared to other known amino acid acylases, A933 acylase seems to be the least thermostable.

The reaction velocity of the enzyme is highest at 37°C (Fig. 4) which is lower than the optimum temperatures for other known amino acid acylases.

pH Stability and pH-Dependence

The assay results in Fig. 5 show that A933 acylase at 37°C is fairly stable in the pH range of 6.5~8.5.

When *N*-chloroacetyl-L-phenylalanine is employed as assay substrate for the L-amino acid acylase activity, optimum deacylation is observed at pH 7.0~7.5 in 40 mM PIPES buffer (Fig. 6). In general, penicillin acylases possess optima in the weakly alkaline pH range for deacylation of penicillins, and in the acidic range for acylation of 6-aminopenicillanate.[20] A933 acylase catalyzes acylation of 6-aminopenicillanate with acyl CoA at pH 7.4 but this activity has not yet been studied in detail.

Divalent Metal Ions

Table 2 summarizes the effect of divalent metal ions on the L-amino acid acylase activity of A933 acylase. It has long been known that amino acid acylases are substantially activated by cobalt ion at concentrations of 1~10 mM. Contrary to our expectation, cobalt ion did not activate, but clearly inhibited, A933 acylase. For example, 2 mM cobalt inhibited the activity of A933 acylase by 96%, whereas it activated acylase I by 300%,[14] suggesting that the L-amino acid acylase activity of A933 acylase is distinct from known amino acid acylases. The results in Table 2 imply that all metal ions, particularly Hg^{++}, Cu^{++}, Zn^{++} and Cd^{++}, are inhibitory to the L-amino acid acylase activity of the acylase.

Specific Inhibitors

Table 3 summarizes the action of *o*-phenanthroline (Fe^{++} chelator), ethylenediaminetetraacetate (EDTA) (chelator) and *p*-chloromercuribenzoate (PCMB) (SH inhibitor) on A933 acylase. At 1 mM, EDTA is the least inhibitory, whereas PCMB completely inactivates the L-amino acid acylase activity, suggesting that A933 acylase may have an active SH group.

Table 2. Effect of divalent metal ions on A933 acylase.

Metal	Concentration (mM)	Relative activity (%)	Metal	Concentration (mM)	Relative activity (%)
None	—	100	Zn^{++}	1	7.7
Hg^{++}	1	0	Fe^{++}	1	53.2
	0.1	6.4	Cd^{++}	1	10.2
	0.01	84.5	Sn^{++}	1	64.4
Cu^{++}	1	0	Ni^{++}	1	79.4
	0.1	87.5	Co^{++}	4	0
Mg^{++}	1	93.6		2	3.8
Mn^{++}	1	74.3		1	82.4
Pb^{++}	1	58.8		0.2	92.3

Table 3. Effects of inhibitors on A933 acylase.

Inhibitor	Concentration (mM)	Relative activity (%)		
		5°C, 15 hours	37°C, 15 minutes	No preincubation
None	—	100	100	100
OPT	1	20.3	34.7	56.4
EDTA	1	80.2	85.4	95.8
PCMB	1	0	0	0

OPT: *o*-Phenanthroline.
EDTA: Ethylenediaminetetraacetate.
PCMB: *p*-Chloromercuribenzoate.

Table 4. Substrate profile of A933 acylase.

Substrate	Relative activity (%)	Substrate	Relative activity (%)
N-Formyl-L-Met	112.7	Hippuryl-L-Phe	1.8
N-Formyl-L-Leu	16.4	L-Leu-L-Leu	8.9
N-Acetyl-Gly	23.9	L-Leu-D-Leu	2.8
N-Acetyl-L-Ala	163.8	D-Leu-L-Leu	1.4
N-Acetyl-D-Ala	0	D-Leu-D-Leu	0
N-Acetyl-L-Val	32.4	Gly-L-Phe	30.2
N-Acetyl-D-Val	0	Gly-D-Phe	1.5
N-Acetyl-L-Leu	92.8	Gly-D-Ala	0.7
N-Acetyl-D-Leu	0	Gly-D-Leu	0
N-Acetyl-L-Met	262.1	L-Ala-Gly	25.8
N-Acetyl-D-Met	0	L-Ala-L-Ala	306.2
N-Acetyl-L-Phe	15.3	β-Ala-L-Ala	1.7
N-Acetyl-D-Phe	0	L-Ser-L-Leu	13.6
N-Acetyl-L-Glu	2.7	Gly-Gly-L-Leu	3.4
N_α-Acetyl-L-Lys	39.8	Gly-Gly-D-Leu	2.7
N-Chloroacetyl-L-Leu	348.5		
N-Chloroacetyl-L-Val	351.1		
N-Chloroacetyl-L-Phe	100.0		

Substrate Profile for Acyl Amino Acids

For objective comparison with known amino acid acylases, the substrate profile of A933 acylase was determined with various acyl amino acids. The results are presented in Table 4, from which it is apparent that A933 acylase belongs to the group of L-amino acid acylases, because it shows no activity toward *N*-acyl-D-amino acids.

Action on Dipeptides and Tripeptides

N-Glycyldipeptides are reported to be susceptible to both hog kidney acylase I[14] (type I in Table 1) and *Aspergillus* acylase[21] (type II in Table 1). As described in a previous paper,[3] L- and D-amino acid acylases from *Pseudomonas* sp. 1158 (type I in Table 1) hydrolyze various dipeptides and tripeptides. For convenient comparison, Table 4 also includes the susceptibility of dipeptides and tripeptides to A933 acylase.

It is interesting to note that, although less stereo-specific, the presence of L-amino acid at the *C*-terminal of peptides seems to result in better susceptibility to A933 acylase, and that L-alanyl-L-alanine is a good dipeptide substrate for A933 acylase, and more susceptible than *N*-acetyl-L-amino acids.

Properties of the A933 Acylase Protein in Blocked Mutant 1501

As described in previous papers,[1,2] *Streptomyces fulvoviridis* A933 1501 is a blocked mutant derived with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) from *S. fulvoviridis* A933 17M9, a producer of PS-5, epithienamycins A and C and MM 17880. The mutant produces the OA-6129 group of carbapenems, because it has no depantothenylating activity. For further characterization of A933 acylase, we have isolated a protein fraction corresponding to A933 acylase protein from blocked mutant 1501 by the same isolation and purification procedure as employed with the parent organism. Enzymological analyses revealed that this protein fraction had the same L-amino acid acylase activity as A933 acylase, but no OA-6129A-depantothenylating activity. If A933 acylase were composed of several units, as is the case with fatty acid synthetase,[6] NTG might have damaged the gene responsible for the OA-6129A-depantothenylating activity, resulting in the shift of the mutant acylase from type III to type II in Table 1.

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