DEACETYLATION OF PS-5, A NEW β-LACTAM COMPOUND*
II. SEPARATION AND PURIFICATION OF L- AND D-AMINO ACID ACYLASES FROM *PSEUDOMONAS* SP. 1158

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(Received for publication March 10, 1980)

L-Amino acid acylase and D-amino acid acylase of *Pseudomonas* sp. 1158 which converted PS-5 to NS-5 (deacetylated PS-5) were separated and purified by sonication, streptomycin and ammonium sulfate fractionations, DEAE-Sephacel column chromatography and gel filtration. Molecular weight and the isoelectric point were estimated to be 75,000 and pI 5.45 for L-amino acid acylase and 100,000 and pI 4.95 for D-amino acid acylase.

In the preceding paper,[1] the results of screening for deacetylation of PS-5 and N-chloroacetylamino acids were reported. Among facultative methanol-assimilating bacteria, *Pseudomonas* sp. 1158 that acted on PS-5 as well as on N-chloroacetyl-L-phenylalanine and N-chloroacetyl-D-valine was chosen for the present study.

KAMEDA *et al.* have recently described that *Pseudomonas* sp. AAA 6020 produced as a single protein DL-amino acid acylase which did not attack N-acetyl amino acids[2]. Coexistence of L-amino acid acylase and D-amino acid acylase has been confirmed by SUGIE and SUZUKI in some species of *Streptomyces*[3,4]. In addition, PS-5 was found to be susceptible to L-amino acid acylase from porcine kidney and D-amino acid acylase from *Streptomyces olivaceus* but not to L-amino acid acylase from *Aspergillus* sp.[1] Under these circumstances, it seemed interesting to determine whether *Pseudomonas* sp. 1158 produced one or more amino acid acylases; and, if more than one enzymes were detected, whether PS-5 was susceptible to some or all of the acylases. Thus we tried to separate and purify the L- and D-amino acid acylase activities of *Pseudomonas* sp. 1158 by sonication, nucleic acid removal with streptomycin, protein fractionation with ammonium sulfate, DEAE-Sephacel column chromatography and gel filtration on Sephadex G-100 and Sephadex G-200.

This paper describes the separation and partial purification of the two types of amino acid acylase together with some physical properties.

Materials and Methods

Materials

N-Chloroacetyl-L-valine and N-chloroacetyl-D-valine were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.). Streptomycin sulfate and enzyme-grade ammonium sulfate were obtained from Toyo Jozo Co., Ltd. (Shizuoka, Japan) and Wako Chemical Industries, Ltd. (Osaka, Japan), respectively. DEAE-Sephacel (1.4 meq/g), Sephadex G-100, Sephadex G-200 and Blue Dextran 2000 were products of Pharmacia Fine Chemicals AB (Uppsala, Sweden). Protein marker kit consisting of cytochrome *c* (horse heart), chymotrypsinogen A (bovine pancreas), albumins (egg white and bovine

* Presented at the 210th Scientific Meeting of Japan Antibiotics Research Association (November 24, 1978).

serum), aldolase (rabbit muscle) and catalase (beef liver) was purchased from Yamanouchi-Boehringer Mannheim Co., Ltd. (Tokyo, Japan). Ampholine (carrier ampholyte pH 3.5~10.0) and a specially designed electrolysis column LKB 8101 (110 ml in capacity) were obtained from LKB-Produkter AB (Bromma, Sweden).

Other reagents were commercially available products of analytical grade.

Microorganism

Pseudomonas sp. 1158, a facultative methanol-assimilating bacterium, was from stock cultures of our laboratories[5] and was cultured as described in the preceding paper[1]. Cells were harvested from the culture broth by centrifugation, washed in a small volume of 0.01 M potassium phosphate buffer, pH 7.4, and suspended in the same buffer solution at a cell density of 80 mg (wet weight)/ml. The average yield of cells was 1.7 g (wet weight)/liter broth. The cell suspension could be stored at −15°C without substantial fall of enzyme activity.

Acylase assay

Activities of L-amino acid acylase and D-amino acid acylase were measured with N-chloroacetyl-L-valine and N-chloroacetyl-D-valine, respectively. The assay substrates were dissolved in distilled water and then adjusted to a pH in the range of 6.5~7.5 with 2 N NaOH. Assay reaction mixture contained, in a final volume of 50 μl, 1 μmole of the substrate (final concentration 20 mM), 50 nmoles of $CoCl_2$ (1 mM), 2.5 μmoles of potassium phosphate buffer, pH 7.4 (50 mM), and enzyme. After incubation at 30°C for 5~30 minutes, the assay reaction mixture was instantly cooled in dry ice-acetone and mixed with 50 μl of 50% acetic acid for termination of deacetylation. The amounts of L-valine and D-valine released were quantitatively analysed with ninhydrin by the method of YEMM-COCKING[6]. One unit of activity was defined to be the amount of enzyme that released 1 μmole of amino acid per hour at 30°C and pH 7.4 from the corresponding N-acylamino acid under the specified assay conditions. The specific activity of enzyme was expressed in units of activity per milligram protein. The quantity of protein was spectrophotometrically measured by the method of KALB, Jr. and BERNLOHR[7].

Disc gel electrophoresis

The original method of DAVIS[8] for polyacrylamide disc gel electrophoresis was employed except that the spacer gel was replaced by 40% sucrose containing enzyme which was directly applied on the coarse gel. Using bromophenol blue as marker, the enzyme protein was subjected to electrophoresis at 4°C and 2 milliamperes per disc (5 mm in diameter). The disc after electrophoresis was longitudinally split into halves. One half of the disc was stained for protein bands with 1% Amino Black 10B in 7% acetic acid. For location of the enzyme activity, the other half was sliced into 1-mm sections each of which was assayed for amino acid acylase activity by using the assay reaction mixture containing 5 μmoles of the substrate, 250 nmoles of $CoCl_2$ and 12.5 μmoles of potassium phosphate buffer, pH 7.4, in a final volume of 0.25 ml. After incubation at 30°C for 60 minutes, the assay reaction mixture was treated for 5 minutes in boiling water and 2 μl of the solution was spotted on a silica gel thin-layer chromatographic plate for amino acid assay[1].

Estimation of the molecular weight

According to the method of ANDREWS[9], the two partially-purified preparations of L- and D-amino acid acylases were subjected to gel filtration on Sephadex G-200. The void volume of a Sephadex G-200 column (2.5×50 cm) was determined with Blue Dextran 2000. The elution volumes of catalase and aldolase were measured by the method of CHANCE *et al.*[10] and that of TAYLOR[11], respectively. The elution peaks of albumins, chymotrypsinogen A and cytochrome *c* were spectrophotometrically located at 280 nm, 280 nm and 415 nm respectively.

Isoelectric focusing in the pH gradient of 3.5~10.0

With 0.8% Ampholine of pH 3.5~10.0, the two types of acylase were subjected to isoelectric focusing at 0~4°C and 300 volts for 48 hours according to the method of VESTERBERG and SVENSSON[12]. After focusing, 1.5-ml fractions were collected from the electrolysis column. The pH of each fraction was measured at 4°C and the enzyme activity was assayed under the standard assay conditions.

Results

Separation and Purification of L- and D-Amino Acid Acylases

Unless otherwise stated, all steps of separation and purification were carried out in a cold room at 0~4°C, using potassium phosphate buffer, pH 7.4, and centrifugation for 30 minutes at 11,000×*g*.

(1) Preparation of crude enzyme extract

The frozen cell suspension (1.2 liters containing 100 g wet cells) was sonicated at 20 kHz (60 W output; UR-200P sonicator, Tomy Seiko Co., Ltd., Tokyo, Japan) for a total period of 15 minutes (15×60-second oscillations with 120-second coolings interposed) and then centrifuged to provide the supernatant solution and the cell debris. The cell debris recovered was again subjected to sonication under the above operational conditions. The combined supernatant solutions showed about 8,000 units of L-amino acid acylase activity and about 3,000 units of D-amino acid acylase activity.

(2) Removal of nucleic acid

Fifty percent streptomycin sulfate solution was added dropwise under gentle stirring to the crude enzyme solution to give a final concentration of 0.4% streptomycin sulfate. The solution was stirred for a further 30 minutes and centrifuged to yield clear enzyme solution.

(3) Protein fractionation with ammonium sulfate

Fine powder of ammonium sulfate was slowly added under agitation to the clear enzyme solution to 60% saturation, while the pH of the solution was maintained in the range of 7.0~7.4 with 4 N NH_4-OH. After stirring for a further 30 minutes, the precipitate was recovered by centrifugation. It was dissolved in 150 ml of the buffer (0.01 M) and dialysed twice in 13 liters each of the same buffer.

(4) Column chromatography on DEAE-Sephacel

Inactive precipitate that formed during dialysis in the retentate was removed by centrifugation. The transparent enzyme solution (170 ml) was charged on a DEAE-Sephacel column (5×60 cm) that had been equilibrated in 0.01 M buffer. After the column was washed with 1 liter of 0.02 M buffer, the

Fig. 1. Separation of L-amino acid acylase and D-amino acid acylase of *Pseudomonas* sp. 1158 on DEAE-Sephacel.

The crude enzyme solution after dialysis was charged on a column (5×60 cm) of DEAE-Sephacel and eluted with a linear concentration gradient of potassium phosphate buffer, pH 7.4, from 0.05 to 0.2 M (10 ml/fraction). The L- and D-acylase activities were assayed with L-chloroacetyl-L- and D-valines, respectively. The protein content was roughly estimated by densitometry at 280 nm.

—○— L-amino acid acylase; —●— D-amino acid acylase.

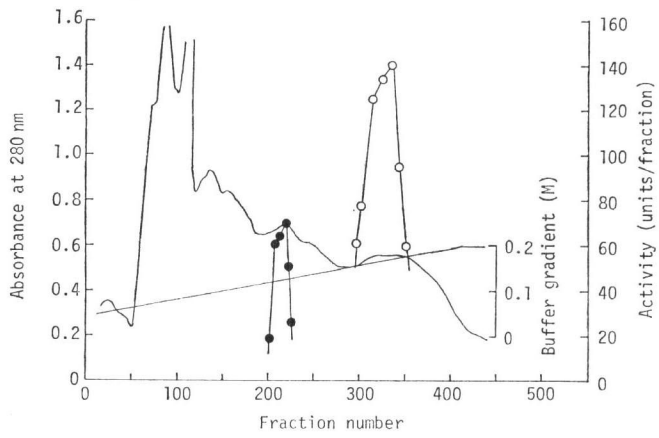


Table 1. Summary of purification of L-amino acid acylase and D-amino acid acylase.

Fraction from	L-Amino acid acylase			D-Amino acid acylase		
	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Cell-free extract	20,252	8,366	0.41	20,252	2,789	0.14
Streptomycin treatment	9,680	6,574	0.68	9,680	3,360	0.35
$(NH_4)_2SO_4$ treatment	4,233	5,528	1.31	4,233	1,479	0.35
DEAE-Sephacel	283	3,712	13.12	110	340	3.09
Sephadex G-100	108	2,498	23.17	71	329	4.62
Sephadex G-200	45	2,807	62.39	44	278	6.25

enzyme activities were eluted with a linear concentration gradient of the buffer from 0.05 M to 0.2 M. Ten-milliliter eluate fractions were collected and assayed for L- and D-amino acid acylase activities. Fig. 1 is a typical elution pattern of L-amino acid acylase and D-amino acid acylase from a DEAE-Sephacel column.

D-Amino acid acylase eluted at a buffer concentration of 0.1 M, whereas L-amino acid acylase appeared at 0.15 M. Active fractions of each enzyme were combined, dialysed in 0.01 M buffer and then freeze-dried.

(5) Gel filtration with Sephadex G-100

The lyophilized preparation of L-amino acid acylase or D-amino acid acylase was dissolved in 2.5 ml of 0.01 M buffer and passed through a Sephadex G-100 column (2×60 cm) that had been bufferized in the same buffer. Active 2.5-ml eluate fractions were combined and condensed to about 5 ml by ultrafiltration with a Diaflo membrane filter PM-10 (Amicon Far East, Ltd., Tokyo, Japan).

(6) Gel filtration with Sephadex G-200

The concentrate of L-amino acid acylase or D-amino acid acylase was further purified by molecular sieving through a Sephadex G-200 column (2.5×50 cm). The amino acid acylase was eluted with 0.01 M buffer and collected in 2.5-ml fractions. Three most active eluate fractions were combined and divided in 0.5-ml portions before storage in a freezer. These partially purified preparations were employed as L-amino acid acylase and D-amino acid acylase in the subsequent physical and enzymological studies.

Table 1 summarizes the yields of L- and D-amino acid acylases during separation and purification. In brief, L-amino acid acylase and D-amino acid acylase were purified 150 and 45 fold from the sonicate respectively.

Physical Properties of L-Amino Acid Acylase and D-Amino Acid Acylase

(1) Disc gel electrophoresis

The purity of the two enzyme preparations was checked by polyacrylamide disc gel electrophoresis followed by protein staining. Their electrophoretic patterns are schematically shown in Fig. 2, using bromophenol blue as marker.

Relative to the marker, the L-amino acid acylase preparation exhibited a major band of Rm 0.57 that was confirmed to act on N-chloroacetyl-L-valine and two minor bands of Rm 0.22 and 0.38 both of which had no enzyme activity. In the D-amino acid acylase preparation, a major protein band of Rm 0.25 having the D-amino acid acylase activity was observed together with three minor bands of no enzyme activity (Rm 0.30, 0.39 and 0.56).

Fig. 2. Disc gel electrophoretic profiles of the partially purified preparations of L-amino acid acylase and D-amino acid acylase from *Pseudomonas* sp. 1158.

For details see the text.

BPB=bromophenol blue

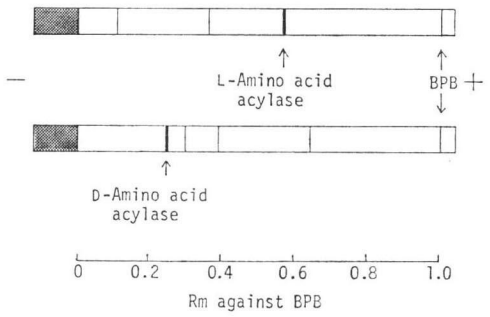
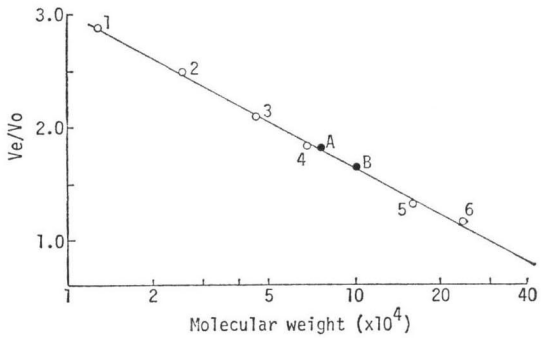


Fig. 3. Molecular weight estimation of L-amino acid acylase and D-amino acid acylase from *Pseudomonas* sp. 1158.

Sephadex G-200 column chromatoghraphy was employed for the molecular weight estimation in comparison with the known molecular weights of the standard proteins. Ve=elution volume, Vo=void volume. (1) cytochrome *c* (M.W. 12,500), (2) chymotrypsinogen A (M.W. 25,000), (3) egg white albumin (M.W. 45,000), (4) bovine serum albumin (M.W. 67,000), (5) aldolase (M.W. 158,000), (6) catalase (M.W. 240,000); (A) L-amino acid acylase, (B) D-amino acid acylase.



(2) Molecular weight

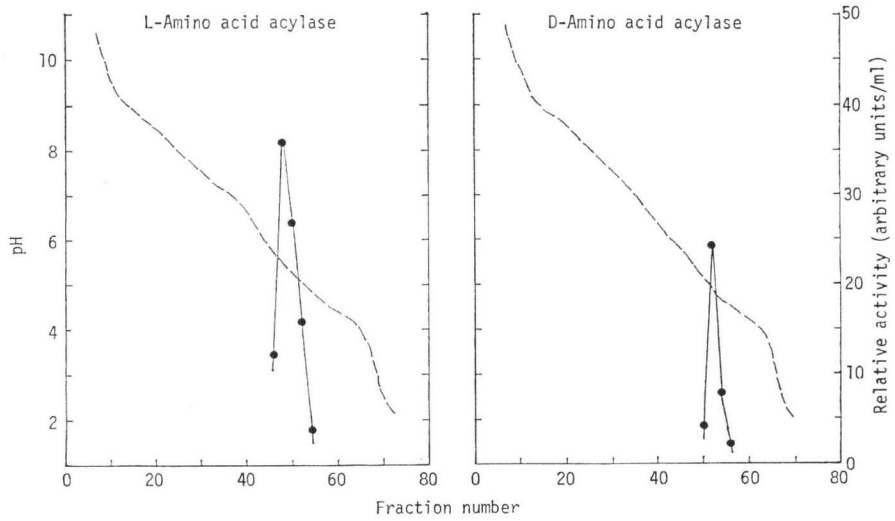
From the elution pattern of the protein marker kit on Sephadex G-200, the molecular weights of L-amino acid acylase and D-amino acid acylase were estimated to be 75,000 and 100,000 respectively (Fig. 3).

(3) Isoelectric point

Isoelectric focusing with Ampholine (pH 3.5~10.0) indicated pI 5.45 for L-amino acid acylase and

Fig. 4. Isoelectric focusing patterns of L-amino acid acylase and D-amino acid acylase in the pH gradient of 3.5~10 (Ampholine).

Partially purified preparations of L-amino acid acylase and D-amino acid acylase from *Pseudomonas* sp. 1158 were subjected to isoelectric focusing at 0~4°C and 300 volts for 48 hours in 0.8% Ampholine of pH 3.5~10.0. After focusing, 1.5-ml fractions were collected for the measurement of pH and the amino acid acylase activity.



pI 4.95 for D-amino acid acylase. There was neither enzymatically active peak other than the relevant amino acid acylase nor mutual contamination in the two preparations. Thus L- and D-amino acid acylases from *Pseudomonas* sp. 1158 were concluded to be an acidic protein (Fig. 4).

Discussion

There are many types of amino acid acylase reported in the literature which deacylate various N-acylamino acids, though their physiological functions are still unknown. For example, acylases I, II and III and N-acetyl-β-alanine deacetylase are found in hog kidney, whereas microorganisms and plants contain L- and D-amino acid acylases. Microbial L- or D-amino acid acylase that is economically important for selective separation of the N-acyl amino acid racemate is distributed in *Aspergillus, Lactobacillus, Micrococcus, Alternaria, Streptomyces* and *Alcaligenes.*

To the best of our knowledge, this is the first paper that describes the coexistence of L- and D-amino acid acylases in one and the same microorganism, although *Pseudomonas* sp. KT83 might have happened to contain the two types of acylases.[13] SUGIE and SUZUKI have recently discovered that some species of *Streptomyces* also produce L- and D-amino acid acylases.[4]

As described in the text, L-amino acid acylase and D-amino acid acylase of *Pseudomonas* sp. 1158 were successfully separated and purified by a combination of anion exchange resin chromatography with gel filtration which was found effective in other amino acid acylases. Enzymological properties of the two types of acylase will be detailed in the subsequent paper.

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