Original Article

Purification of Extracellular Aryl Acylamidase from a Coryneform Bacterium, Strain A-1*

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A coryneform bacterium, strain A-1, produced extracellularly an aryl acylamidase, which was markedly activated by preincubation with a substrate, acetanilide (AAN). This specific enzyme was purified by ammonium sulfate fractionation under the coexistence of AAN, followed by chromatographic fractionations with DEAE-Sephadex A-50 and Sephadex G-100. The enzyme purified 263-fold over crude preparation gave a single band on SDS-PAGE. The molecular weight was estimated to be *ca.* 127,000, which was appreciably larger than those of known bacterial aryl acylamidases. The activated enzyme tended to be more tolerant of high temperatures and some metal ions, compared with the nonactivated one. This purified and activated enzyme also hydrolyzed aniline-based pesticides such as naproanilide, chlorpropham, propanil and linuron. Kinetic analysis of substrate-saturation curves of these chemicals showed that the compound's overall ability to act as a substrate was higher for chemicals having unsubstituted aniline moiety than for ones having Cl-substituted aniline moiety in the molecule. It was strongly suggested that the enzyme conformation was induced to fit readily for unsubstituted acylanilides due to the activation with AAN.

INTRODUCTION

In a previous study,¹⁾ we isolated soil bacteria capable of producing aryl acylamidases extracellularly and examined some properties of the extracellular crude enzymes. Such active bacteria were universally found in soils and enzymes from about half the bacteria were markedly activated by preincubation with a substrate, acetanilide (AAN). It was supposed that the activation mechanism could be explained in terms of the induced-fit of enzymes for AAN.

This paper describes the purification of aryl acylamidase from a coryneform bacterium, strain A-1, and characterizes its enzymic properties and substrate specificity in order to make the activation by substrate clear.

MATERIALS AND METHODS

1. Chemicals and Materials

Acetanilide (AAN), *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED), isopropyl *N*-(3-chlorophenyl)carbamate (chlorpropham), 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea (linuron) and cellophane tubing-seamless 20/32 inch for dialysis (cellophane tube) were purchased from Wako Pure Chemical. 3',4'-Dichloropropionanilide (propanil) and 2-(β -naphthyloxy)propionanilide (naproanilide) were donated by Hodogaya Chemical and Mitsui Toatsu Chemicals, respectively. Ehrlich's meat extract and peptone were purchased from Kyokuto Seiyaku Kogyo. DEAE-

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Sephadex A-50, Sephadex G-100 and Blue Dextran 2000 were purchased from Pharmacia LKB. Cytochrome C from horse heart, albumin from chicken egg and albumin from bovine serum were purchased from Sigma Chemical and lactate dehydrogenase from pig heart from Oriental Yeast. Other reagents and materials were purchased from Wako Pure Chemical or Nakalai Chemicals. Aniline (AN) for calibration was used after distillation.

2. Bacterial Strain and Crude Enzyme Preparation

A coryneform bacterium, strain A-1, isolated from a paddy field soil in a previous study¹⁾ was employed. The strain was precultured at 30°C for 3 to 5 days in 10 ml of Bouillon medium containing 1% Ehrlich's meat extract, 1% peptone and 0.5% NaCl in distilled water. The preculture broth was added to 3 l of Bouillon medium and the broth was left standing at 30°C for 9 days with occasional stirring. Crude enzyme preparation was obtained by centrifuging the broth at $11,000 \times g$ at 0°C for 20 min.

3. Activation Treatment of Enzyme

Each ten milliliters of the enzyme preparation packed into a cellophane tube was immersed in 2 l of 18.5 mM AAN at 5°C for 96 hr with gentle stirring. The inner solution was dialyzed twice against 2 l of distilled water at 5°C for 12 hr each with gentle stirring in order to remove AAN and/or AN, and this was employed as activated enzyme preparation.

4. Assay of Enzyme Activity and Protein Content

AN released from AAN during incubation was determined by diazotization and the coupling method.¹⁾ A typical procedure is as follows: One milliliter of the enzyme preparation was mixed with 1.0 ml of 0.1 M Na-phosphate buffer (pH 7.0) and 2.0 ml of 37 mM AAN, and the reaction mixture was incubated at 25°C for 1.0 hr with reciprocal shaking. To the mixture, 1.0 ml of 1 N HCl, 4.2 ml of distilled water and 0.2 ml of 2% NaNO₂ were added. After occasional stirring for 5 min, 0.2 ml of 10% sulfamic acid was added, and the mixture was left standing for 10 min with

occasional stirring. After 0.4 ml of 1% NED was added, the mixture was again left standing for 2 hr. Released AN was estimated colorimetrically by measuring the absorbance at 550 nm.

One unit (U) of the enzyme activity was defined as the amount of enzyme releasing 1.0 μ mol of AN per minute.

The protein contents in enzyme solutions were determined by the method of Lowry *et al.*²⁾ with bovine serum albumin as a standard protein.

5. Purification of Enzyme

All steps for purification were carried out at 5°C. The first fractionation with ammonium sulfate was done under the coexistence of AAN to prevent enzyme denaturation.

5.1 Ammonium sulfate fractionation

Three liters of the crude enzyme preparation was combined with 3 l of 37 mM AAN. After gentle stirring for 6 hr, ammonium sulfate was added to 30% saturation. After further gentle stirring overnight, the precipitate was collected by centrifuging at $11,000 \times g$ for 20 min, dissolved in 250 ml of 0.1 M Na-phosphate buffer (pH 7.0) and dialyzed against distilled water for 24 hr. The enzyme activity was determined after activation as described above.

5.2 Ion-exchange chromatography

Twenty grams of DEAE-Sephadex A-50 was equilibrated with 0.2 M phosphate buffer (pH 7.0), washed with 0.02 M phosphate buffer (pH 7.0), and packed into a column ($5\phi \times 100$ cm). A 200-ml portion of the fractionated sample was then applied to the column at a flow rate of 0.85 ml/min. The adsorbed enzyme was eluted by a linear gradient with 0.02 M phosphate buffer (pH 7.0) and the same buffer containing 1.0 M NaCl at the same flow rate. Fractions having the activity were pooled and the enzyme was precipitated by adding ammonium sulfate to 30% saturation under the coexistence of 18.5 mM AAN. The precipitate was dissolved in 30 ml of 0.1 M Na-phosphate buffer (pH 7.0), dialyzed against distilled water for 24 hr, and activated again as described above.

5.3 Gel filtration

Ten grams of Sephadex G-100 was equilibrated with 0.2 M phosphate buffer (pH 7.0),

washed with 0.02 M phosphate buffer (pH 7.0), and packed into a column ($2\phi \times 120$ cm). Each 3.0-ml portion of the above sample was then applied and the enzyme was eluted with 0.02 M phosphate buffer (pH 7.0) at a flow rate of 0.65 ml/min. Fractions having the activity were pooled, dialyzed against distilled water for 24 hr, concentrated by freeze-drying and activated again as described above.

5.4 Polyacrylamide gel electrophoresis (PAGE)

PAGE was carried out with 7.5% polyacrylamide gel (pH 8.3) by Nagai's technique⁸⁾ based on the method of Ornstein⁴⁾ and Davis.⁵⁾ Each 0.2-ml portion of the enzyme sample and a drop of glycerol were applied on the gel and migrated at a constant current of 3 mA per gel tube for about 30 min. Bromophenol Blue was used as a tracking dye and the gel was stained with Amido-Black 10B in the usual way.

6. Estimation of Molecular Weight of Enzyme

The molecular weight of the purified enzyme was estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with cytochrome C from horse heart, albumin from chicken egg, albumin from bovine serum and lactate dehydrogenase from pig heart as standard proteins.

SDS-PAGE was carried out with 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) by Hayashi & Ohba's technique⁶⁾ that is a modification of Weber & Osborn's method.⁷⁾ The standard proteins (*ca.* 60 μ g each) for calibration were dissolved in 1 ml of 0.02 M Na-phosphate buffer (pH 7.2) containing 50% glycerol, 2% SDS and 2% mercaptoethanol, and combined with 2.5 ml of

0.05% Bromophenol Blue as a tracking dye. A 100- μ l portion of the solution was applied on the gel with a drop of glycerol and migrated at a constant current of 8 mA per gel tube for 2 hr. The gel was stained with Amido-Black 10B in the usual way. The purified enzyme preparation was migrated in the same manner and the molecular weight was estimated from the calibration curve.

7. Estimation of Substrate Specificity

Propanil, naproanilide, chlorpropham and linuron besides AAN were employed. The enzyme activity was estimated by determining the corresponding ANs, unsubstituted AN, 3-chloro-AN or 3,4-dichloro-AN released during incubation by diazotization and the coupling method.⁹⁾ Activities of all chemicals were compared at a concentration of 0.50 μ M. *K_m* and *V_{max}* values were determined as best-fit values with a computer on AAN, propanil, chlorpropham and linuron having solubility of over 75 ppm in water.

RESULTS AND DISCUSSION

1. Enzyme Purification and Molecular Weight Estimation

It was found in preliminary studies that extracellular aryl acylamidase from a coryneform bacterium, strain A-1,¹⁾ was denatured with ammonium sulfate, and that the denaturation was prevented effectively under the coexistence of AAN. Therefore, the crude enzyme preparation was preincubated with AAN for 6 hr, followed by ammonium sulfate fractionation, ion-exchange chromatography with DEAE-Sephadex A-50 and gel filtration with Sephadex G-100.

Table 1 Purification of aryl acylamidase from a coryneform bacterium, strain A-1.

Fractionation step	Total protein (mg)	Total activity ^{a)} (10^{-3} U)	Specific activity (10^{-3} U/mg)	Purification (fold)	Activity yield (%)
Crude enzyme solution ^{b)}	960.00	20,000	20.8	1	100
Ammonium sulfate fractionation ^{c)}	34.75	14,166	407.6	20	71
DEAE-Sephadex A-50 chromatography	3.74	14,688	3927.3	189	73
Sephadex G-100 gel filtration	2.40	13,140	5475.0	263	66

^{a)} Activity after activation treatment with AAN. Unit (U) = μ mol of aniline released/min.

^{b)} Supernatant of liquid culture broth.

^{c)} Fraction of 30% saturation.

Fig. 1 Estimation of the molecular weight of the enzyme by SDS-polyacrylamide gel electrophoresis.

The data on purification are summarized in Table 1. Although the enzyme had been partially activated by the preincubation with AAN, the activity in each step of the purification was determined after reactivation. The enzyme was purified 263 fold to a specific activity of 5.475 U/mg protein from the crude enzyme preparation with a yield of 66%, and the purified enzyme gave a single band in PAGE.

The purified enzyme was a monomer which gave a single band in SDS-PAGE and its molecular weight was estimated to be approximately 127,000 as shown in Fig. 1.

The molecular weight of this enzyme was much larger than reported weights of any other bacterial aryl acylamidases (approximately 52,500–80,000) from *Pseudomonas acidovorans*,⁹⁾ *P. fluorescens*,¹⁰⁾ *P. alcaligenes*,¹¹⁾ *P. aeruginosa*,¹²⁾ *Bacillus sphaericus*¹³⁾ and *Escherichia coli*.¹⁴⁾

2. Properties of Activated Enzyme

By employing both crude and purified enzyme preparations immediately after activation, effects of pH, temperature, metal ions and EDTA on enzyme reaction were examined.

Effects of pH and temperature on the activity and stability of the purified enzyme preparation are shown in Fig. 2. The optimal pH and temperature of enzyme reaction were 7.0 and 35°C, respectively, and the enzyme was

Fig. 2 Effects of pH and temperature on the activity (○) and stability (●) of the purified enzyme.

Stability was tested by exposing the enzyme to various pH and temperatures for 1 hr prior to the assay at 25°C. One unit (U) of the enzyme activity was defined as the amount of enzyme releasing 1.0 μ mol of aniline per minute.

Table 2 Effects of metal ions and EDTA on the activity of crude and purified enzymes after activation.

Chemical ^{a)}	Relative activity (%)	
	Crude enzyme	Purified enzyme
None	100	100
KCl	101	100
Li ₂ SO ₄	100	100
CaCl ₂	118	104
MnCl ₂	95	93
ZnSO ₄	71	93
CoCl ₂	94	85
MgCl ₂	87	93
FeCl ₂	91	85
CuCl ₂	34	19
AgNO ₃	8	11
EDTA	14	18

^{a)} The concentration of each chemical in the reaction mixture was 2.5 mM.

fairly stable in the pH 6.5 to 8.0 region and unstable during incubation (1 hr) at over 35°C. These findings were similar to those of the crude enzyme preparation.

Effects of metal ions and EDTA at 2.5 mM are shown in Table 2. The activities of both preparations were slightly inhibited by Mn²⁺,

Zn²⁺, Co²⁺, Mg²⁺ and Fe²⁺. Sulfhydryl inhibitors such as Ag⁺ and Cu²⁺ were effective. K⁺ and Li⁺ had no effects but Ca²⁺ slightly enhanced the activity. EDTA, a chelating agent, was obviously inhibitory, which suggested that a certain metal ion was essential for the enzyme.

Compared with the properties of the non-activated enzyme described in our previous paper,¹⁾ the activated enzyme became more tolerant of high temperatures and metal ions such as Mn²⁺, Zn²⁺, Co²⁺, Mg²⁺ and Fe²⁺. It seemed that activation tended to make the enzyme stable.

3. Compounds for Enzyme Substrates

As shown in Table 3, the purified enzyme exhibited broad substrate specificity and hydrolyzed not only acylanilides such as AAN, propanil and naproanilide, but also phenylcarbamate chlorpropham and phenylurea linuron. Judging from the activity values at a substrate concentration of 0.50 μ M, naproanilide and AAN were most degradable with the enzyme, followed by chlorpropham. Propanil and linuron were slightly degraded. Kinetic parameters, K_m and V_{max} , were determined on these pesticides, except naproanilide with extremely low water-solubility (ca. 2.5 μ M), and their apparent first-order rate constants (V_{max}/K_m) were obtained, as shown in Table 3. It is wellknown that the apparent

first-order rate constant is proportional to the rate of hydrolysis at low substrate concentrations, and that it represents a compound's overall ability to act as a substrate for hydrolase.^{15,16)} Actually, the apparent first-order rate constants obtained here were proportional to the aryl acylamidase activities at a low substrate concentration. As for the ability as enzyme substrate, the compounds with unsubstituted AN moiety were superior to the ones with Cl-substituted AN moiety in the molecule. It was strongly suggested that the enzyme was induced to fit easily for unsubstituted acylanilides because it had been activated with AAN.

It is of interest whether this bacterial extracellular enzyme could be activated by other chemicals than AAN. If this is the case, this enzyme might play an important role in degrading a variety of pesticides with an AN moiety in the fields.

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Table 3 Degradation of some aniline-based pesticides by the activated and purified enzyme at pH 7.0 and 35°C.

Chemical	Activity ^{a)} (μ mol/ mg protein/ min)	K_m (M)	V_{max} (μ mol/ mg protein/ min)	First- order rate constant (V_{max}/K_m)
	$\times 10^{-3}$	$\times 10^{-3}$		$\times 10^8$
AAN	7.9	0.35	7.11	20.3
Propanil	1.4	0.38	0.79	2.1
Naproanilide	9.3	ND ^{b)}	ND	ND
Chlorpropham	3.4	0.10	1.01	10.1
Linuron	1.9	0.24	0.79	3.3

^{a)} The concentration of each chemical in the reaction mixture was 0.50 μ M.

^{b)} Not determined.

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要 約

Coryneform 細菌 A-1 株の産生する菌体外 aryl acylamidase の精製*

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土壌からの分離細菌 A-1 株は coryneform グループ

に属し, 基質アセトアニリド (AAN) とのインキュベーション下で著しく活性化される特異な aryl acylamidase を産生した. 本酵素は AAN 共存下での硫酸分画, イオン交換クロマトグラフィーおよびゲル濾過によって 263 倍まで精製され, その SDS-PAGE は同種の既知細菌産生酵素よりかなり大きい分子量 (127,000) をもつ単一バンドを与えた. 精製活性化酵素は温度や幾つかの金属イオンに対する安定性を増すとともに, napro-anilide, chlorpropham, propanil および linuron に対しても加水分解活性を示した. これらの基質飽和曲線の酵素反応論的解析は, 分子内のアニリン部位が無置換体であるものの方が塩素置換体であるものより基質として適していることを示した. これは AAN による活性化で, 酵素の構造が無置換体に対してより適合するように変化したためと考えられた.

* 土壌 aryl acylamidases に関する研究 (第2報)