Aliphatic Nitrile Hydratase from *Arthrobacter* sp. J-1 Purification and Characterization†

Yasuhisa ASANO, Kinya FUJISHIRO, Yoshiki TANI and Hideaki YAMADA

Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan

Received August 10, 1981

A new enzyme, aliphatic nitrile hydratase, which hydrates acetonitrile to form acetamide was purified from the cell-free extract of acetonitrile-grown *Arthrobacter* sp. J-1. The overall purification was about 290-fold with a yield of 10%. The purified enzyme was homogeneous as judged by ultracentrifugation and disc gel electrophoresis. The enzyme catalyzed the stoichiometric hydration of acetonitrile to form acetamide according to the following scheme: $CH_3CN+H_2O \rightarrow CH_3CONH_2$. The enzyme was inducibly formed and then amidase which hydrolyzed acetamide was formed. The molecular weight of the enzyme was determined to be about 420,000 by gel filtration. The enzyme was composed of two kinds of subunits, of which the molecular weights were 24,000 and 27,000. The isoelectric point was 3.6. The enzyme was active toward low molecular weight aliphatic nitriles of 2 to 5 carbon atoms. The *Km* value for acetonitrile was determined to be 5.78 mM. The enzyme was inactivated by sulfhydryl reagents. The enzyme was competitively inhibited by potassium cyanide: the *Ki* value was 1.5 μM.

Since Thimann and Mahadevan[1,2] demonstrated that nitrilase (EC 3.5.5.1) purified from barley leaves catalyzed the hydrolysis of indoleacetonitrile to indoleacetic acid and ammonia, several nitrilases have been found and characterized.[3~6] Nitrilases have been found to hydrolyze nitriles to the corresponding carboxylic acid and ammonia without forming amide as an intermediate, and do not use amide as substrate. However, a small but constant amount of amide was detected in the reaction mixture of partially purified ricinine nitrilase (EC 3.5.5.2).[4] On the other hand, the formation of amides during the growth of microorganisms on aliphatic nitriles has been reported by several workers.[7~12] A question that has remained unsolved is whether the enzymatic hydrolysis of an aliphatic nitrile is catalyzed by more than one enzyme.[17,18]

In the course of investigation on the microbial degradation of nitrile compounds,[11~15] we found a new enzyme, which catalyzed the hydrolysis of acetonitrile to form ammonia through acetamide in cooperation with amidase, in *Arthrobacter* sp. J-1. The enzyme was apparently different from known nitrilases. Thus, the nitrilase activity of the organism was proved to be due to a combination of nitrile hydratase and amidase.[13]

The present paper describes the formation of nitrile hydratase and amidase, and purification and enzymological properties of the former enzyme from *Arthrobacter* sp. J-1.

MATERIALS AND METHODS

Materials. Hydroxyapatite was prepared according to the method of Tiselius *et al.*[19] Dimethylsuberimidate dihydrochloride was purchased from Wako Pure Chemicals Ltd., Osaka. The standard proteins used for the molecular weight determination were purchased from Boeringer Mannheim GmbH and Pharmacia Fine Chemicals. Amidase was partially purified from acetonitrile-grown *Arthrobacter* sp. J-1[10] (Step 5 of the purification procedure)[16] and was confirmed to be free of nitrile hydratase activity. Other chemicals were commercial products.

† Microbial Degradation of Nitrile Compounds. Part V. For Part IV, see ref. 15.

Microorganisms and cultivation. Arthrobacter sp. J-1,[10] which could grow on acetonitrile as the sole source of carbon and nitrogen, was used. The culture medium contained 3.9 g acetonitrile, 13.4 g K_2HPO_4, 6.5 g KH_2PO_4, 1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 μg biotin, 0.4 mg calcium pantothenate, 2 mg inositol, 0.4 mg nicotinic acid, 0.4 mg pyridoxine·HCl, 0.4 mg *p*-aminobenzoic acid, 0.2 mg riboflavin and 0.01 mg folic acid in 1 liter of tap water, pH 7.0. Cells were aerobically grown at 28°C. Cell growth was estimated turbidimetrically: 0.65 mg dry cell weight per ml was equivalent to 1.0 unit of OD_{610}.

Assay methods. Nitrile hydratase activity was routinely assayed by measuring the production of ammonia from nitriles in the presence of amidase. The standard reaction mixture contained 50 μmol of potassium phosphate buffer, pH 7.0, 5 μmol of acetonitrile, 0.11 units of amidase and enzyme solution in a total volume of 0.5 ml. The reaction was started by addition of acetonitrile and carried out at 30°C for 20 min. The amount of ammonia was measured by the indophenol method.[20] In this assay system, the enzyme activity was evaluated properly in proportion to the protein concentration of nitrile hydratase, when more than 0.1 units of amidase was added to the reaction mixture. The reaction was found to be linear with time and enzyme concentration.

To estimate the amount of acetamide, the reaction was carried out in the absence of amidase and terminated by addition of 0.05 ml of 1 N HCl. The reaction mixture was applied to Shimadzu gas-liquid chromatograph, Model GC-4CM, equipped with a flame ionization detector. The column used was a glass column of 3 mm inside diameter, 1 m length, packed with Porapack Q (80 to 100 mesh). Column, injection and detector temperatures were 210°C, 240°C and 240°C, respectively. The carrier gas was N_2 at 40 cm^3/min. The integration and calibration of peak areas were carried out with a Shimadzu Chromatopack C-R1A.

Amidase activity was measured by the production of ammonia in the reaction mixture containing 50 μmol of potassium phosphate buffer, pH 7.0, 5 μmol of acetamide and enzyme solution in a total volume of 0.5 ml. The reaction was carried out for 20 min at 30°C. The amount of ammonia was measured by the indophenol method.[20]

One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of ammonia or amide per min.

Analytical methods. Protein was assayed by the method of Lowry *et al.*[21] or from the absorbance at 280 nm, using an $E^{1\%}_{1\,cm}$ value of 26.6 which was obtained from dry weight determination. The purity and sedimentation coefficient measurements were performed with a Spinco model E analytical ultracentrifuge.

Mass spectra were measured with a Hitachi M-70 gas chromatography-mass spectrometer (GC-MS). The column used was a glass column of 3 mm inside diameter, 1 m length, packed with Tenax GC. The operational conditions were: injection temperature, 242°C; column temperature, 193°C; interface temperature, 248°C; chamber temperature, 185°C; ionization voltage, 20 eV; carrier gas, He at 14 cm^3/min.

The molecular weight of the enzyme was estimated by the method of Andrews,[22] using a 0.9 × 115 cm column of Sepharose 6B with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The molecular weight of the subunit was determined by polyacrylamide gel electrophoresis according to the method of Weber and Osborn.[23]

Isoelectric focusing. Isoelectric focusing was performed according to the method of Vesterberg.[24] Electrophoresis was carried out at 5°C for 70 hr using Ampholite (LKB Produkter) of a pH range of 4 to 6.

RESULTS

Formation of nitrile hydratase and amidase by Arthrobacter sp. J-1

Figure 1 shows the change in the specific activities of nitrile hydratase and amidase in *Arthrobacter* sp. J-1 during growth on acetonitrile. The specific activity of nitrile hydratase was high in the early logarithmic phase, around 12-hr cultivation, and thereafter rapidly decreased. The specific activity of ami-

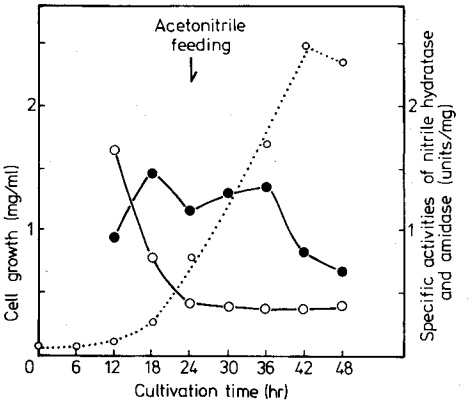


FIG. 1. Formation of Nitrile Hydratase and Amidase during Cultivation of *Arthrobacter* sp. J-1 on Acetonitrile.
Cultivation was carried out at 28°C in a 100-liter jar fermentor with 70 liters of the basal medium, under aeration of 35 liter/min and agitation at 280 rpm. Initial concentration of acetonitrile was 0.4%, and 0.4% of acetonitrile was added to the medium at 24 hr-cultivation. The activity was assayed by measuring ammonia formation. Nitrile hydratase (○), amidase (●) and growth (--○--).

TABLE I. SPECIFIC ACTIVITIES OF NITRILE HYDRATASE AND AMIDASE IN CELL-EXTRACT OF *Arthrobacter* sp. J-1

Washed cells after 2-days cultivation* (33 mg) were inoculated into 500 ml of the basal medium containing growth substrates at the indicated concentrations (w/v). Cultivation was carried out in 2-liter shaking flasks. The activities in the cell-free extract were assayed by measuring ammonia formation.

Growth substrate	Cultivation time (hr)	Specific activity (milli-units/mg)	
		Nitrile hydratase	Amidase
Acetonitrile (0.4%)	24	100	300
Acetamide (0.5%)	24	170	210
Sodium acetate (0.5%) +ammonium sulfate (0.2%)	48	13	89
n-Butyronitrile (0.4%)	36	96	7.2
Benzonitrile (0.1%)	48	0.30	2.0
Glucose (0.5%)+ ammonium sulfate (0.2%)	48	9.0	1.0
Sodium succinate (0.5%) +ammonium sulfate (0.2%)	24	3.0	5.4

* The cell-free extract of the seed cells had 90 milli-units/mg of nitrile hydratase and 450 milli-units/mg of amidase activities.

dase reached maximum at 18 hr. The concentration of acetamide in the culture broth was maximum (about 10 mM) around 12-hr cultivation and then decreased to zero at 18 hr.

The effects of carbon and nitrogen sources on the activities of nitrile hydratase and amidase were examined. Table I shows that nitrile hydratase was inducibly formed when the strain was grown on acetonitrile, acetamide and *n*-butyronitrile. Amidase was also formed when it was grown on acetonitrile, acetamide and acetate.

Purification of nitrile hydratase

All enzyme purification procedures were performed at 0 to 5°C and with potassium phosphate buffer, pH 7.0.

Step 1. Preparation of cell-free extract. Washed cells from 60 liters of culture were suspended in the buffer (0.1 M) and disrupted for 120 min on ice with a Kaijo-denki 19 kHz ultrasonic oscillator. The disrupted cells were centrifuged at $14,000 \times g$ for 20 min.

Step 2. Protamine sulfate treatment. To the resultant supernatant was added 5% protamine sulfate solution, to a final concentration of 0.1 g protamine per 1 g protein. After 30 min, the precipitate formed was removed by centrifugation at $14,000 \times g$ for 20 min. The supernatant was dialyzed against the buffer (0.01 M).

Step 3. DEAE-cellulose column chromatography. The dialyzed solution was applied on a DEAE-cellulose column (10.5×20 cm) equilibrated with the buffer (0.01 M). After washing the column with the buffer (0.05 M) containing 0.2 M NaCl, the enzyme was eluted with the same buffer containing 0.35 M NaCl.

Step 4. Ammonium sulfate fractionation. Solid ammonium sulfate was added to the eluate (760 ml) to 40% saturation (185 g). After stirring for 30 min, the mixture was centrifuged at $14,000 \times g$ for 30 min, and then solid ammonium sulfate was added to the supernatant to 60% saturation. The active precipitate was dissolved in a small volume of 0.01 M buffer and dialyzed against the same buffer.

Step 5. First hydroxyapatite column chromatography. The dialyzed enzyme solution was

placed on a hydroxyapatite column (5.2 × 13.5 cm) equilibrated with the buffer (0.01 M). The enzyme was eluted with a linear concentration gradient from 0.01 M to 0.06 M in the buffer. The active fractions (519 ml) were concentrated by adding solid ammonium sulfate to 80% saturation. The precipitate collected by centrifugation was dissolved in a small volume of the buffer (0.01 M) and dialyzed against the same buffer.

Step 6. Second hydroxyapatite column chromatography. The dialyzed enzyme solution was again applied on a hydroxyapatite column (4.9 × 6 cm) and eluted in a similar manner. The active fractions (414 ml) were concentrated by ultrafiltration.

Steps 7 and 8. Sephadex G-200 column chromatographies. The concentrated enzyme solution was placed on a column of Sephadex G-200 (3.5 × 110 cm) equilibrated with the buffer (0.05 M) containing 0.1 M NaCl. The active fractions eluted with the same buffer were pooled and concentrated by ultrafiltration. This step was carried out twice.

A summary of purification of nitrile hydratase shows that about 290-fold purification with a 10% yield from the cell-free extract was achieved (Table II).

TABLE II. SUMMARY OF PURIFICATION OF NITRILE HYDRATASE FROM *Arthrobacter* sp. J-1

The activity was assayed by measuring ammonia formation. The amount of protein was determined from the absorbance at 280 nm, except for in the cell-free extract and protamine sulfate steps.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Cell-free extract	19,900	638	0.032
Protamine sulfate	10,200	528	0.052
DEAE-cellulose	377	423	1.12
Ammonium sulfate (40~60%)	232	513	2.21
1st Hydroxyapatite	52	298	5.70
2nd Hydroxyapatite	29	166	5.71
1st Sephadex G-200	12	84	7.28
2nd Sephadex G-200	7	63	9.17

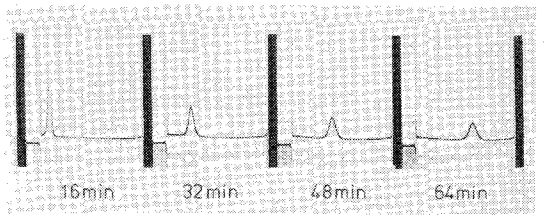


FIG. 2. Sedimentation Boundaries of the Purified Enzyme.

Sedimentation was done at 42,040 rpm in sodium phosphate buffer (pH 7.0). Sedimentation is from left to right.

FIG. 3. Polyacrylamide Gel Electrophoresis of the Purified Enzyme.

Purified enzyme (150 μg) was mixed with 36 μmol of triethanolamine-NaOH buffer (pH 8.2) and 900 μg of dimethylsuberimidate dihydrochloride in a total volume of 240 μl and incubated at room temperature for 12 hr. The treated enzyme (6 μg) was subjected to electrophoresis at a current of 5 mA.

TABLE III. IDENTIFICATION OF REACTION PRODUCT BY GAS-LIQUID CHROMATOGRAPHY

The reaction was performed under the standard conditions. Nitrile hydratase (2.91 μg) was used.

	t_R (min)			
	Column*			
	A	B	C	D
Reaction product	20.5	10.9	17.8	14.2
Acetamide	20.1	10.9	17.7	14.3

* Columns used and other operational conditions were:

A: Porapack Q (80 to 100 mesh), 3 mm × 2 m; column temperature, 170°C; injection and detector temperature, 200°C.

B: Tenax GC (80 to 100 mesh), 3 mm × 2 m; column temperature, 150°C; injection and detector temperature, 200°C.

C: Thermon-1000 + H_3PO_4 (5 + 0.5%) on Chromosorb W (80 to 100 mesh), 3 mm × 2 m; column temperature, 120°C; injection and detector temperature, 150°C.

D: Thermon-1000 + H_3PO_4 (10 + 0.5%) on Chromosorb W (80 to 100 mesh), 3 mm × 2 m; column temperature, 140°C; injection and detector temperature, 170°C.

Purity of nitrile hydratase

The purified enzyme was sedimented as a single symmetric peak on ultracentrifugation as shown in Fig. 2. Assuming a partial specific volume of 0.75, the sedimentation coefficient ($s_{20,w}$) was estimated to be 15.4 S at the enzyme concentration of 0.25%. Figure 3 shows the results of disc gel electrophoresis of the purified enzyme after treatment with dimethylsuberimidate, which is a specific cross-linking reagent between protomers of a protein.[25] The enzyme showed a single band on the gel.

Identification of reaction product

Identification of the reaction product with nitrile hydratase was performed with acetonitrile as substrate by gas-liquid chromatography. As shown in Table III, the retention times of the reaction product were identical with those of authentic acetamide.

Stoichiometry of acetonitrile hydration

The stoichiometry of acetonitrile consumption and acetamide formation during the hydration of acetonitrile was examined by gas-liquid chromatography. As shown in Fig. 4, it was found that 1 mol of acetamide was produced per 1 mol of acetonitrile consumed. The enzyme did not hydrolyze acetonitrile directly to acetic acid and ammonia, and did not have amidase activity. Based on the results, the enzyme was proved to catalyze the following reaction: $CH_3CN + H_2O \rightarrow CH_3CONH_2$.

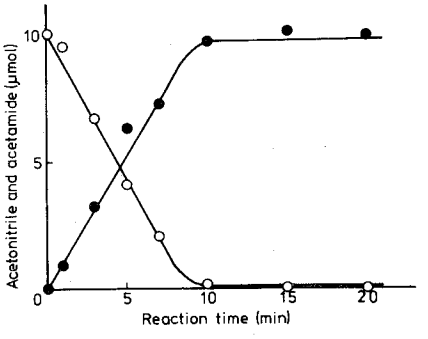


FIG. 4. Time Course of Acetonitrile Hydration by the Enzyme.

The reaction mixture contained 1 unit of the enzyme, 10 μmol of acetonitrile and 100 μmol of potassium phosphate buffer (pH 7.0). The concentrations of acetonitrile and acetamide were determined by gas-liquid chromatography. Acetonitrile (○) and acetamide (●).

Molecular weight and isoelectric point

The molecular weight of the native enzyme was calculated to be approximately 420,000 by

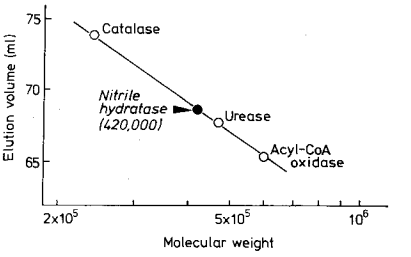
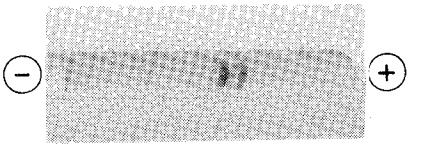


FIG. 5. Determination of Molecular Weight of the Enzyme by Gel Filtration on Sepharose 6B.

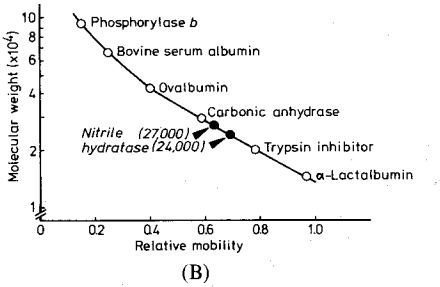
Experimental conditions are described in MATERIALS AND METHODS. The molecular weights of reference proteins are: catalase, 240,000; urease, 480,000; and acyl-CoA oxidase, 600,000.[26]



(A)

FIG.6(A). SDS-Disc Gel Electrophoresis of the Purified Enzyme.

Purified enzyme was incubated in the presence of 1% SDS and 3% 2-mercaptoethanol at 95°C for 3 min. The enzyme (12 μg) was subjected to electrophoresis in the presence of 0.1% SDS at a current of 8 mA per tube. The gels were stained with Coomassie Brilliant Blue R-250.



(B)

FIG. 6(B). Determination of Molecular Weight of Subunits of the Enzyme by SDS-disc Gel Electrophoresis.

The experimental conditions are as described in Fig. 2(A). The molecular weights of reference proteins are: α-lactalbumin, 14,400; trypsin inhibitor, 20,100; carbonic anhydrase, 30,000; ovalbumin, 43,000; bovine serum albumin, 68,000; and phosphorylase *b*, 94,000.

gel filtration on Sepharose 6B (Fig. 5). The enzyme gave two bands on sodium dodecylsulfate (SDS)-disc gel electrophoresis as shown in Fig. 6. The molecular weights of the subunits were calculated to be 24,000 and 27,000, and the ratio of each absorption at 600 nm was 41 : 59, as measured with a densitometer.

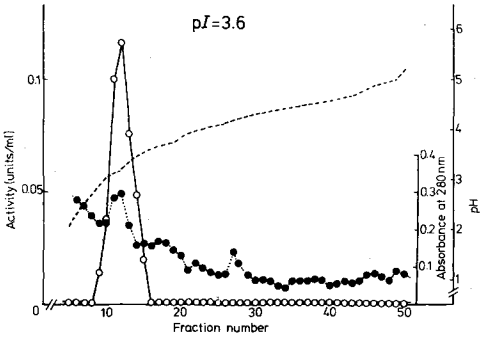


FIG. 7. Isoelectric Focusing of the Enzyme.

The enzyme (0.188 mg) was used. The enzyme activity was measured by amide formation with gas-liquid chromatography. Absorbance at 280 nm (●) and enzyme activity (○).

The enzyme had an isoelectric point of pH 3.6 as shown in Fig. 7.

Absorption spectrum

The absorption spectrum of the purified enzyme in 0.01 M potassium phosphate buffer, pH 7.0, showed the maximum absorbance at 280 nm, with a small shoulder at 290 nm.

Effect of temperature and pH on the enzyme activity

The optimum temperature for the enzyme activity was found to be 35°C. The activation energy below 35°C was calculated to be 9580 cal/mol. Eighty percent of the activity was retained after incubation at 35°C for 10 min, whereas the enzyme was nearly completely inactivated at 55°C for 10 min. The enzyme exhibited the maximal activity at pH 7.0～7.2 and was stable around pH 6. Glycine-NaOH buffer activated the enzyme and potassium phosphate buffer had a negative effect on the enzyme stability.

TABLE IV. SUBSTRATE SPECIFICITY OF THE ENZYME

The reaction conditions were the same as described in MATERIALS AND METHODS except that various nitriles or amides were used as substrates in place of acetonitrile or acetamide.

Substrate	Structure	Relative* activity (%)	*Km* (mM)
Chloroacetonitrile	$ClCH_2CN$	130	10.9
Acetonitrile[a]	CH_3CN	100	5.78
Propionitrile[a]	CH_3CH_2CN	81.0	1.90
n-Butyronitrile	$CH_3CH_2CH_2CN$	59.8	10.8
n-Valeronitrile	$CH_3CH_2CH_2CH_2CN$	2.4	—
Hydroxyacetonitrile[b,c]	$HOCH_2CN$	30.0	—
Acrylonitrile[a]	$CH_2=CHCN$	25.5	0.88
Methacrylonitrile	$CH_2=C(CH_3)CN$	15.5	8.77
Methoxyacetonitrile[b,d]	CH_3OCH_2CN	10.0	—
α-Cyanoacetamide	H_2NOCCH_2CN	5.3	—
Malononitrile	$NCCH_2CN$	4.5	—

* Relative initial velocity at 30°C.
The enzyme activities toward nitriles except for those marked "*a*" and "*b*" were assayed by amide formation with gas-liquid chromatography. The enzyme activities toward amides (except for α-cyanoacetamide) were assayed by ammonia formation without addition of amidase.

[a] Assayed by ammonia formation with addition of amidase.

[b] Assayed by substrate consumption with gas-liquid chromatography.

[c] The reaction product was identified as hydroxyacetamide by GC-MS analysis. The spectrum revealed ion peaks at *m/z* 75 $[M]^+$, 73 $[M^+ - H_2]$, 44 $[O{\equiv}CNH_2]^+$ (base peak).

[d] The reaction product was identified as methoxyacetamide by GC-MS analysis. The spectrum revealed ion peaks at *m/z* 90 $[M+1]^+$, 89 $[M]^+$, 70, 59 $[M^+ - CH_2O]$ (base peak), 44 $[CONH_2]^+$

TABLE V. EFFECT OF METAL IONS AND INHIBITORS ON THE ENZYME

The enzyme activity was measured by amide formation with gas-liquid chromatography, after the enzyme had been preincubated for 10 min at 30°C with various metal ions or inhibitors at the concentration indicated.

Metal (1 mM)	Relative rate (%)	Inhibitor (1 mM)	Relative rate (%)
None	100	None	100
LiCl	96	NaN_3	101
NaCl	91	HCN (0.1 mM)	0
$AgNO_3$	0	Hydroxylamine (10 mM)	98
$MgCl_2$	96	*o*-Phenanthroline	96
$CaCl_2$	100	α,α′-Dipyridyl	97
$BaCl_2$	85	8-Oxyquinoline	98
$MnCl_2$	112	EDTA	98
$ZnCl_2$	92	5,5′-Dithio-bis(2-nitrobenzoic acid)	100
$CdCl_2$	92	*N*-Ethylmaleimide	112
$SnCl_2$	97		
$PbCl_2$	99	Iodoacetic acid	23
$NiCl_2$	104	*p*-Chloromercuribenzoate	59
$CuCl_2$	99	Urea	97
$HgCl_2$ (0.01 mM)	0	Acrylamide	118
$FeCl_3$	95		
$AlCl_3$	94		

Substrate specificity

The enzyme activity toward various nitriles was examined at a concentration of 10 mM. Table IV shows that chloroacetonitrile, acetonitrile, propionitrile, *n*-butyronitrile, hydroxyacetonitrile, acrylonitrile and methacrylonitrile were significantly active as substrates for the enzyme. Aliphatic nitriles with more than five carbon atoms, aromatic nitriles and various amides tested were inactive as substrates. These included isobutyronitrile, capronitrile, crotonitrile, lactonitrile, succinonitrile, glutaronitrile, adiponitrile, 2,4-dicyano-1-butene, benzonitrile, benzylcyanide, potassium cyanide, α-cyanohydrin, β-cyano-L-alanine, formamide, acetamide, acrylamide, propionamide, *n*-butyramide, isobutyramide, methacrylamide, *n*-valeramide, succinamide, benzamide, phenylacetamide and lactamide. The enzyme did not catalyze the hydrolysis of nitriles or amides to carboxylic acids and ammonia for all nitriles or amides tested.

Effect of metal ions and inhibitors

The effect of metal ions and inhibitors at 1 mM concentration on the enzyme activity is shown in Table V. The enzyme activity was strongly inhibited by sulfhydryl reagents such as Ag^+, Hg^{2+}, iodoacetate and *p*-chloromercuribenzoate. The inhibition by Hg^{2+} was much stronger than that by *p*-chloromercuribenzoate as shown in Table VI. The inhibition by these inhibitors was counteracted by ad-

TABLE VI. EFFECT OF SH-INHIBITORS ON THE ENZYME ACTIVITY WITH OR WITHOUT 2-MERCAPTOETHANOL

The enzyme activity was measured as described in Table V except that the enzyme was preincubated for 10 min at 30°C in a reaction mixture containing various SH-inhibitors and/or 1 mM 2-mercaptoethanol.

SH-Inhibitor	Concentration (mM)	2-Mercaptoethanol (1 mM)	
		−	+
None		100%	98%
$HgCl_2$	0.001	34	105
	0.01	0	101
p-Chloromercuribenzoate	0.1	88	98
	1.0	59	94
	5.0	16	67
Iodoacetate	1.0	23	105

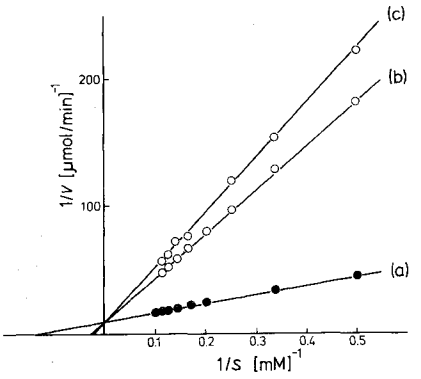


FIG. 8. Inhibition of the Enzyme by Potassium Cyanide.
Potassium cyanide was added to the reaction mixture at concentrations of 0 mM (a), 0.005 mM (b) and 0.01 mM (c). The enzyme activity was assayed by ammonia formation, with acetonitrile as a substrate. Amidase was not inhibited by potassium cyanide at these concentrations.

dition of 2-mercaptoethanol.

Potassium cyanide competitively inhibited the enzyme activity and the enzyme inhibition was recovered by overnight dialysis. As shown in Fig. 8, the inhibition was competitive with acetonitrile as substrate. The *Ki* value was 1.5 μM. The enzyme was inhibited by isobutyronitrile and glutaronitrile, which presumably act as substrate analogs. urea, acrylamide, propionamide, *n*-butyramide, methacrylamide, benzamide and ammonia did not inhibit the enzyme activity at 10 mM.

Stability

The enzyme was stable on storage at $-20°C$ in the presence of 50% glycerol for at least 40 days at pH 7.0 and about 60% of the activity remained after 6 months storage. Half of the initial activity was lost on storage at 0°C for 40 days, pH 7.0.

DISCUSSION

Nitrile hydratase which was inducibly formed in the cells of acetonitrile-grown *Arthrobacter* sp. J-1 was purified. The enzyme was characterized to catalyze the formation of acetamide from acetonitrile. No activity to hydrolyze acetonitrile directly to acetic acid and ammonia or amidase activity was detected.

Nitrilases which degrade nitriles to the corresponding carboxylic acid and ammonia have been well-characterized.[1~6] However, an enzyme that forms amide by the hydration of a nitrile is not reported except for a few instances of conversion of specific nitriles. Enzymes which catalyzed the hydration of β-cyanoalanine and toyocamycin were found in blue lupin[27] and in *Streptomyces rimosus*[28] producing the antibiotic, respectively. The hydration of potassium cyanide was also shown by formamide hydro-lyase.[29] These enzymes have not been purified to homogeneity and not characterized well. None of them were active toward low molecular weight aliphatic nitriles.

The enzyme in the present paper was active on low molecular weight aliphatic nitriles, forming the corresponding amides, and no carboxylic acid and ammonia were detected. The enzyme was completely different from known nitrilases[1~6] and other hydrolases[28,29] acting on nitrile groups in the reaction mode and substrate specificity. Therefore, we propose that the new enzyme catalyzing the formation of amide from nitrile should be called "aliphatic nitrile hydratase," because nitrilase is defined to catalyze the direct hydrolysis of nitrile to carboxylic acid and ammonia:

$$\text{R-CN} \xrightarrow[\text{Nitrilase}]{2H_2O} \text{R-COOH} + NH_3$$

$$\text{R-CN} \xrightarrow[\substack{\text{Nitrile}\\\text{hydratase}}]{H_2O} \text{R-CONH}_2 \xrightarrow[\text{Amidase}]{H_2O} \text{R-COOH} + NH_3$$

The inhibition by potassium cyanide, which is an inhibitor of enzymes having a metal, carbonyl or persulfide group at the active center,[30,31] was peculiar in this enzyme. The inhibition was restored by overnight dialysis and was the competitive type with a *Ki* value of 1.5 μM. There has been no report showing that cyanide acts as a competitive inhibitor, even concerning enzymes with a nitrile as substrate. The enzyme was not inactivated by metal chelating or carbonyl reagents, but strongly inactivated by sulfhydryl reagents. These re-

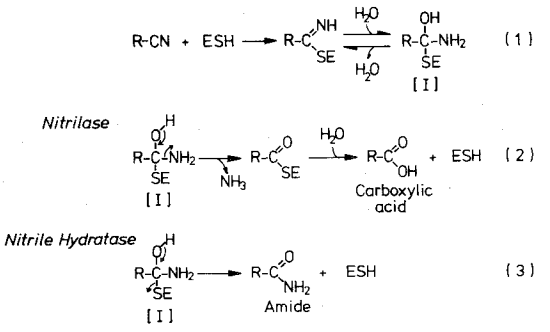


FIG. 9. Proposed Reaction Mechanisms of the Enzyme.

sults indicate that the enzyme has neither a metal nor a carbonyl group at the active center but a reactive sulfhydryl group.

Considering the mechanisms for nitrile hydrolysis by acid or base[32] and the existence of a reactive sulfhydryl group in the enzyme, proposed reaction mechanisms for nitrilase and nitrile hydratase are shown in Fig. 9. A mechanism of nitrile hydrolysis catalyzed by nitrilase, which was first proposed by Hook and Robinson,[4] is presented in equations (1) and (2). A nucleophilic attack by a reactive sulfhydryl group of the enzyme on the nitrile carbon forms an enzyme-bound imine, which is hydrated to form a tetrahedral intermediate [I]: An acyl-enzyme is formed from the intermediate [I], with ammonia as a leaving group. The acyl-enzyme is then hydrolyzed to form a carboxylic acid. The reaction of nitrile hydratase in the present study which forms only amide would proceed as shown in equations (1) and (3), in which the tetrahedral intermediate [I] decomposes to yield amide, with the enzyme as a leaving group. The enzyme produced only amide without accompanying carboxylic acid or ammonia from all substrates tested. A small but constant amount of amide was produced through the same pathway by ricinine nitrilase.

Harper showed in *Nocardia rhodochrous*[5] and *Fusarium solani*[6] that benzonitrile was directly hydrolyzed to benzoic acid and ammonia by nitrilase. *Arthrobacter* sp. J-1 also decomposed benzonitrile likewise, as it grows on benzonitrile as a sole source of carbon and nitrogen.[33] These results suggest that there are at least two distinct pathways for nitrile hydrolysis, regardless of the species. One is a pathway in which both nitrile hydratase and amidase are involved, and the other is a pathway in which only nitrilase is involved. The chemical hydrolysis of acetonitrile, propionitrile and benzonitrile proceeds exclusively *via* amide.[34~36] Whether there is a correlation between the kind of nitrile and the hydrolytic enzyme or not would be of interest in enzymology. It may be possible to say that nitriles with saturated alkyl groups, such as acetonitrile, are hydrated with nitrile hydratase followed by hydrolysis of the amide by amidase. On the other hand, as acrylonitrile is degraded directly to carboxylic acid and ammonia in *Arthrobacter* sp. I-9,[11] it may be possible to say that nitriles can be directly hydrolyzed to carboxylic acid and ammonia if the nitrile group is conjugated with a double bond(s) like in acrylonitrile and benzonitrile.

The enzyme was active toward low molecular weight aliphatic nitriles with 2 to 5 carbon atoms. As the alkyl group of a nitrile becomes longer, the enzyme becomes less active toward the substrate. Chloroacetonitrile was the best substrate for the enzyme. The electron-withdrawing effect of the chlorine atom seems to favor the nucleophilic attack of the sulfhydryl group of the enzyme on the nitrile carbon. However, malononitrile, which also has a strong electron-withdrawing group, a nitrile group, was not so active as chloroacetonitrile.

Further accumulation of knowledge on the microbial degradation of nitriles is necessary to clarify the relationship between the structure of nitriles and the degradation pathways.

Acknowledgments. We thank Mr. M. Tachibana for his skillful assistance. We are also grateful to Dr. T. Ueno, Pesticide Research Institute, Kyoto University, for his help with gas chromatography-mass spectrometry. This work was supported in part by a Grant-in-Aid for Environmental Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1) K. V. Thimann and S. Mahadevan, *Arch. Biochem. Biophys.*, **105**, 133 (1964).
2) S. Mahadevan and K. V. Thimann, *Arch. Biochem. Biophys.*, **107**, 62 (1964).
3) W. G. Robinson and R. H. Hock, *J. Biol. Chem.*, **239**, 4257 (1964).
4) R. H. Hook and W. G. Robinson, *J. Biol. Chem.*, **239**, 4263 (1964).
5) D. B. Harper, *Biochem. J.*, **165**, 309 (1977).
6) D. B. Harper, *Biochem. J.*, **167**, 185 (1977).
7) A. Mimura, T. Kawano and K. Yamaga, *J. Ferment. Technol.*, **47**, 631 (1969).
8) J. L. Firmin and D. O. Gray, *Biochem. J.*, **158**, 223 (1976).
9) M. J. DiGeronimo and A. D. Antoine, *Appl. Environ. Microbiol.*, **31**, 900 (1976).
10) M. Kuwahara, H. Yanase, Y. Ishida and Y. Kikuchi, *J. Ferment. Technol.*, **58**, 573 (1980).
11) H. Yamada, Y. Asano, T. Hino and Y. Tani, *J. Ferment. Technol.*, **57**, 8 (1979).
12) H. Yamada, Y. Asano and Y. Tani, *J. Ferment. Technol.*, **58**, 495 (1980).
13) Y. Asano, Y. Tani and H. Yamada, *Agric. Biol. Chem.*, **44**, 2251 (1980).
14) Y. Asano, S. Ando, Y. Tani and H. Yamada, *Agric. Biol. Chem.*, **44**, 2497 (1980).
15) Y. Asano, S. Ando, Y. Tani, H. Yamada and T. Ueno, *Agric. Biol. Chem.*, **45**, 57 (1981).
16) Y. Asano, M. Tachibana, Y. Tani and H. Yamada, *Agric. Biol. Chem.*, **46**, 1175 (1982).
17) A. Arnaud, P. Galzy and J. C. Jallageas, *Agric. Biol. Chem.*, **41**, 2183 (1977).
18) J. C. Jallageas, A. Arnaud and P. Galzy, *J. Gen. Appl. Microbiol.*, **24**, 103 (1978).
19) A. Tiselius, S. Hjertén and Ö. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1956).
20) J. K. Fawcett and J. E. Scott, *J. Clin. Path.*, **13**, 156 (1960).
21) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
22) P. Andrews, *Biochem. J.*, **91**, 222 (1964).
23) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
24) O. Vesterberg, "Methods in Enzymology," Vol. XXII, ed. by W. B. Jacoby, Academic Press Inc., N. Y., 1971, p. 389.
25) G. E. Davis and G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.*, **66**, 651 (1970).
26) S. Shimizu, K. Yasui, Y. Tani and H. Yamada, *Biochem. Biophys. Res. Commun.*, **91**, 108 (1979).
27) P. A. Castric, K. J. F. Farnden and E. E. Conn, *Arch. Biochem. Biophys.*, **152**, 62 (1972).
28) T. Uematsu and R. T. Suhadolnik, *Arch. Biochem. Biophys.*, **162**, 614 (1974).
29) W. E. Fry and R. L. Millar, *Arch. Biochem. Biophys.*, **151**, 468 (1972).
30) E. J. Hewitt and D. J. D. Nicolas, "Metabolic Inhibitors," Vol. II, ed. by R. M. Hochster and J. H. Quastel, Academic Press Inc., N. Y., 1963, p. 311.
31) R. C. Bray, "The Enzymes," Vol. XII, ed. by P. D. Boyer, Academic Press Inc., N. Y., 1975, p. 299.
32) M. L. Kilipatric, *J. Am. Chem. Soc.*, **69**, 40 (1947).
33) Y. Asano, K. Fujishiro, Y. Tani and H. Yamada, in preparation.
34) B. S. Rabinovitch, C. A. Winkler and A. R. P. Stewart, *Can. J. Res.*, **B20**, 185 (1942).
35) B. S. Rabinovitch and C. A. Winkler, *Can. J. Res.*, **B20**, 221 (1942).
36) Y. Ogata and M. Okano, *J. Chem. Soc. Jpn.*, **70**, 32 (1949).