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Formation and Purification of Nitrile Hydratase from *Corynebacterium pseudodiphteriticum* ZBB-41

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ABSTRACT

A strain of nitrile hydratase-forming microorganism, *Corynebacterium pseudodiphteriticum* ZBB-41, was isolated from soil and the conditions for the enzyme formation have been studied. Addition of ferric or ferrous ions and the use of methacrylamide as an inducer greatly enhanced enzyme formation. When the strain was cultivated at 27°C for 64 h in an optimum medium, the specific activity of the enzyme was 83U/mg of dry cell weight. The enzyme was purified from the crude extract of methacrylamide induced cells in 6 steps with 9.5-fold purification and 8.8% recovery. The enzyme has a molecular mass of 80,000 and consists of two subunits with molecular mass of 28,000 and 25,000 respectively. The isoelectric point was 4.2. The Km value to acrylonitrile was 0.21 *M*. The enzyme was strongly inhibited by Hg^{2+}, Cu^{2+}, Ag^{+}, iodoacetate, and phenyl mercuric acetate and can be protected by 2-mercaptoethanol. The enzyme was noncompetitively inhibited by sodium cyanide, the Ki value was 0.13 m*M*.

Index Entries: Nitrile hydratase; purification; *Corynebacterium pseudodiphterititicum;* bioconversion.

INTRODUCTION

Several kinds of aliphatic nitrile hydratase such as in *Pseudomonas chlororaphis* B-23 (*1*), *Arthrobacter* sp J-1 (2), and *Corynebacterium hoffmanii* (3) have been reported. The enzymes catalyzed the stoichiometric hydration

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of nitriles to form amides according to the following scheme: $R-CN+H_2O-R-CONH_2$. The enzymes were inducibly formed when the strains were grown on suitable nitriles or amides as nitrogen sources and/or inducers.

Some investigators had described the purification and characterization of aliphatic nitrile hydratase from *Arthrobacter* sp J-1 (4),*Pseudomonas chlororaphis* B-23 (5), and *Brevibacterium* R312 (6). However, there is very little information on aliphatic nitrile hydratase from some strains of *Corynebacterium*. The nitrile hydratase was partially purified from the cells of *Corynebacterium pseudodiphteriticum* ZBB-41 grown on the butyronitrile in the laboratory, but the purification and characterization of the enzyme have yet to be carried out. This report describes the formation, purification, and characterization of nitrile hydratase from the new source.

MATERIALS AND METHODS

Microorganism and Cultivation

The *Corynebacterium pseudoditeriticum* ZBB-41, isolated from soil and selected as the highest acrylamide-producing strain in the laboratory (3), was used. It was cultivated at 28°C for 64 h in a basal medium consisting of K_2HPO_4 2 g, NaCl 1 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, thiamine 0.4 mg, biotin 2 μg, inositol 2 mg, and $FeSO_4 \cdot 7H_2O$ 10 mg with various amounts of carbon and nitrogen sources as indicated in each experiment, per L of tap water, pH 7.5.

Chemicals

Acrylamide and methacrylamide were purchased from Aldrich (USA). Acrylonitrile was purified by redistillation before it was used as a substrate for enzyme assay. DEAE-cellulose DE-52, Sephadex G-200, and standard proteins were obtained from Pharmacia (Sweden). Ampholyte was from Bio-Red (USA). The Hydroxyapatite and all other chemicals were commercial products.

Biomass

The biomass was estimated turbidimetrically by a dry cell calibration curve of the absorbance of A_{460nm}; 1.0 U of $A_{460nm}=0.07$ mg of dry cell weight/mL.

Enzyme Assay

The nitrile hydratase activity was assayed by measuring the amount of acrylamide produced from acrylonitrile. An appropriate amount of enzyme (or culture broth) was incubated in 5.0 mL of 0.05 *M* potassium phosphate buffer (pH 7.5) containing 0.5 m*M* acrylonitrile at 25°C for 10

Table 1
Effect of Carbon Sources on Biomass and Formation of Nitrile Hydratase

Carbon source	Biomass, mg/mL	Enzyme activity, U/mL	Specific activity, U/mg
Glucose	4.00	5.9	1.5
Fructose	0.10	0.4	4.1
Maltose	2.97	16.9	5.7
Sucrose	2.79	16.5	5.9
Lactose	2.95	13.6	4.6
Mannitol	2.66	18.9	7.1
Glycerol	3.76	7.9	2.1
Starch	2.66	14.4	5.4
Dextrin	2.54	24.7	9.7
None	2.66	13.0	4.9

min. The reaction was stopped by addition of 0.1 mL of 1.0 *M* HCl. The acrylamide formed was assayed by HPLC (Waters, USA) (*7*). One unit of enzyme activity was defined as the amount of the enzyme that catalyzed the formation of 1.0 μM acrylamide/min under the assay conditions.

Analytical Methods

Protein was assayed by the Coomassie Brilliant Blue G-250 dye-binding method of Bradford (*8*), with bovine serum albumin as standard. Polyacrylamide disc gel electrophoresis (PAGE) was carried out in 7.5 and 12.5% gels in Tris-HCl buffer (pH 8.9) according to the method of Davis (*9*). SDS-gel electrophoresis (SDS-PAGE) was carried out in 10% polyacrylamide slab gels by the method of King (*10*). The relative molecular mass of the subunits of the enzyme were determined from the relative mobilities of standard proteins. The isoelectric focusing of the enzyme was performed according to the method of Vesterberg (*11*).

RESULTS AND DISCUSSION

Effect of Carbon Sources on Enzyme Formation

The strain was cultivated respectively in the basal medium containing 0.5% (w/v) of various carbon sources and 0.5% (w/v) of butyronitrile at 28°C for 64 h. The estimations of biomass and enzyme activity in the intact cells (Table 1) showed that dextrin was the best carbon source for biomass and enzyme formation. When dextrin was used as a carbon source, its optimum concentration was 1.0%.

Table 2
Effect of Nitriles and Amides on Biomass and Formation of Nitrile Hydratase

Inducer	Biomass, mg/mL	Enzyme activity, U/mL	Specific activity, U/mg
Acetonitrile	1.90	0.43	0.2
Propionitrile	2.38	11.90	5.0
n-Butyronitrile	2.66	18.50	7.0
iso-Butyronitrile	2.51	25.10	10.0
Glutaronitrile	0.13	0	0
Acrylonitrile		no growth	
Adiponitrile		no growth	
Acetamide	1.70	4.60	2.7
Propionamide	2.30	11.00	4.7
n-Butyramide	2.50	21.00	8.4
iso-Butyramide	2.50	31.00	12.4
Methacrylamide	3.10	39.30	12.8
None	0.75	0	0

Inducibility of Enzyme

The nitrile hydratase of *C. pseudodiphteriticum* ZBB-41 was inducibly formed when *n*-butyronitrile was used as an inducer. But *n*-butyronitrile is volatile and partly lost under aerobic conditions. Therefore, an attempt was made to look for an inducer that could replace *n*-butyronitrile. Various nitriles and amides (0.5% w/v) were tested. As shown in Table 2, acetonitrile, propionitrile, *n*- and iso-butyronitrile could induce the formation of nitrile hydratase. No better inducer than iso-butyronitrile (also volatile) was found among the nitriles tested. Table 2 also shows that the conversion products of *n*- and iso-butyronitrile, that is, *n*- and iso-butyramide, also induced the enzyme formation with higher specific activity than that of the corresponding substrates. Methacrylamide, in particular, was the most powerful inducer of the nitrile hydratase.

Effect of Inorganic Compounds on Enzyme Formation

The effect of various inorganic compounds on the formation of nitrile hydratase was examined with the basal medium containing 1.0% dextrin and 0.5% methacrylamide without any metal ions. As shown in Table 3, $FeSo_4$ and $FeCl_3$ greatly increased enzyme activity. The optimum concentration of $FeSO_4 \cdot 7H_2O$ was 10 mg/L. This result suggests that the enzyme required ferrous or ferric ion as a necessary cofactor. The addition of equal amounts of KH_2PO_4 and K_2HPO_4 in the medium could greatly increase the biomass, but does not benefit enzyme formation.

Table 3
Effect of Inorganic Compounds on Biomass and Formation of Nitrile Hydratase

Inorganic compounds	Concentration, mg/mL	Biomass, mg/mL	Enzyme activity, U/mL	Specific activity, U/mg
$CaCl \cdot 2H_2O$	6.5	1.20	56	47
$MgSo_4 \cdot 7H_2O$	10.0	1.85	60	33
$NiSO_4 \cdot 7H_2O$	9.0	0.40	21	50
$FeSO_4 \cdot 7H_2O$	5.0	1.10	59	53
	10.0	1.10	62	62
$FeCl_3$	6.5	1.00	52	52
KH_2PO_4/	500/500	4.10	39.8	9.7
K_2HPO_4	1000/1000	4.10	41.1	10.0
None		0.88	24.0	27.0

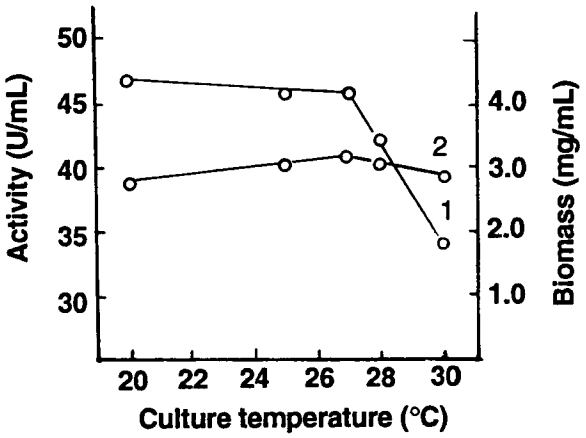

Fig. 1. Effect of temperature on formation of nitrile hydratase. 1. Enzyme activity; 2. Biomass.

Effect of pH and Temperature on Enzyme Formation

The optimum conditions for enzyme formation were pH 7.5–8.0 and culture temperature 20–27°C. When temperature was higher than 27°C, the enzyme activity was sharply decreased. (Fig. 1).

Time Course of Enzyme Formation

The highest enzyme activity appeared when the strain was cultivated for 64 h, although the greatest biomass was reached at 50 h during the growth of the bacteria (Fig. 2). Based on the above results, a medium (1 L

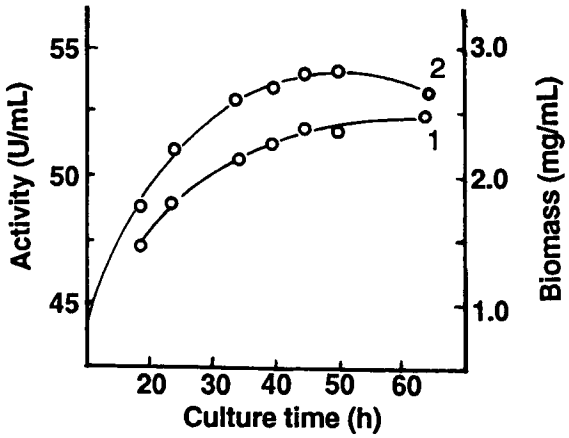

Fig. 2. Time course for formation of nitrile hydratase.1. Enzyme activity; 2. Biomass.

of tap water) containing dextrin 10 g, methacrylamide 5 g, K_2HPO_4 2 g, NaCl 1 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, thiamine 0.4 mg, biotin 2 μg, inositol 2 mg, cysteine 0.5 g, and $FeSO_4 \cdot 7H_2O$ 10 mg (pH 7.5), seems to be most suitable for the formation of nitrile hydratase from *C. pseudodiphteriticum* ZBB-41. When the strain was cultivated at 27°C for 64 h in this meidum, the specific activity of the enzyme was about 83 U/mg dry cell.

Purification of Nitrile Hydratase

Purification of the enzyme was carried out from crude extract of the cells induced with methacrylamide. In order to stabilize the enzyme activity, all of the operations were performed at a temperature near 4°C.

Step 1. Preparation of crude extract: The washed cells were suspended in 0.1 *M* potassium phosphate buffer, pH 7.0, and disrupted for 9 min on ice with an ultrasonic oscillator (Labsonic 2000, B.Braun, Sweden) at 19 KHz, 200 W. The cell debris was removed by centrifugation at 14,000×g for 30 min.

Step 2. Ammonium sulfate fractionation: Solid ammonium sulfate was added to the crude extract to 30% saturation. The pH was maintained at 7.0 with ammonia. The precipitate was removed by centrifugation. Solid ammonium sulfate was added to 75% saturation. After about 4 h, the precipitate was collected by centrifugation and dissolved in a small amount of 0.01 *M* potassium phosphate buffer, pH 7.0, and then dialyzed against the same buffer.

Step 3. DEAE-Cellulose DE-52 column chromatography: The desalted sample from step 2 was applied to a column of DEAE-cellulose (2.6×25 cm) that had been equilibrated with 0.05 *M* potassium phosphate buffer, pH 7.0. The enzyme was first eluted with 0.05 *M* potassium phosphate buffer containing 0.2 *M* NaCl at 30 mL/h. When the eluant volume reached

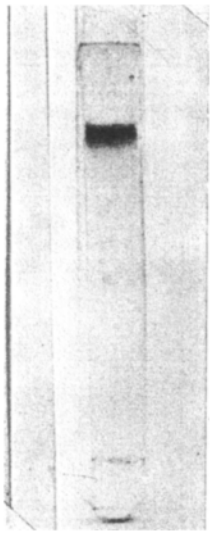

Fig. 3. Polyacrylamide get electrophoresis of the purified nitrile hydratase.

about 100 mL, the enzyme was eluted further with the same buffer containing 0.35 *M* NaCl at the same flow rate and 4 mL fractions were collected. The activity fractions of the nitrile hydratase were pooled and concentrated with solid ammonium sulfate and dialyzed against 0.01 *M* potassium phosphate buffer, pH 7.0

Step 4. First hydroxyapatite column chromatography: The desalted sample from step 3 was applied to a hydroxyapatite column (2.6×10 cm) equilibrated with 0.01 *M* phosphate buffer, pH 7.0. The enzyme was eluted with a linear gradient (0.01 *M*–0.06 *M*) of phosphate buffer, pH 7.0, at a flow rate of 30 mL/h and 15 mL fractions were collected. The further procedures were the same as step 3.

Step 5. Second hydroxyapatite column chromatography: The dialyzed sample from step 4 was applied again on a second hydroxyapatite column (1.6×15 cm) and then eluted as in step 4.

Step 6. Sephadex G-200 column chromatography: The active fractions from step 5 were applied to a column of Sephadex G-200 (1.6×75 cm) equilibrated with 0.05 *M* phosphate buffer containing 0.1 *M* NaCl, pH 7.0, and eluted with the same buffer at a flow rate of 8 mL/h and 4 mL fractions were collected. Those fractions with the enzyme activity were pooled.

By gel electrophoresis using 10% gel, this enzyme preparation was found to contain one protein band with nitrile hydratase activity (Fig. 3). The purification of nitrile hydratase from *C. pseudodiphteriticum* ZBB-41 is summarized in Table 4. The enzyme was purified 9.5-fold with a recovery of 8.8% and a final specific activity of 315.2 U/mg protein. The poor recovery of the enzyme may be due to the instability of the enzyme.

Table 4
Purification of Nitrile Hydratase from *C. pseudodiphteriticum* ZBB-41*

Purification step	Total protein, mg	Total activity, U	Specific activity, U/mg protein	Purification factor	Recovery, %
Crude extract	1080	35,700	33.1	1.0	100
Ammonium sulfate (30–75%)	618	31,500	51.4	1.6	88.2
DEAE-Cellulose DE-52	132	18,640	141.2	4.3	52.2
First hydroxyapatite	71	13,840	194.9	5.9	38.8
Second hydroxyapatite	40	8,000	200.0	6.1	22.4
Sephadex G-200	10	3,152	315.2	9.5	8.8

*From 9 L of culture broth.

General Properties

The molecular mass of nitrile hydratase was estimated to be about 80,000 by a gradient gel electrophoresis with 7.5% and 12.5% gel (Fig. 4). As shown in Figure 5, the enzyme gave two protein bands on SDS-gel electrophoresis with 10% gel. The molecular mass of the subunits were about 25,000 and 28,000 respectively. These results suggested that nitrile hydratase may be an oligomer consisting of three subunits. The isoelectric point of the enzyme was 4.2 as estimated by polyacrylamide gel isoelectric focusing.

The optimum temperature for the enzyme activity was 25°C, about 40% of the initial activity was retained after incubation at 20°C for 90 min, but lost activity at 50°C for 90 min. The enzyme exhibited maximum activity at pH 7.5 and was stable in the pH range of 6.5–8.0 after incubation at 25°C for 90 min.

Effect of Metal Ions and Inhibitors

The nitrile hydratase was strongly inhibited by Hg^{2+}, Cu^{2+}, Ag^{+}, iodoacetate, phenyl mercuric acetate, and EDTA (Table 5) and can be protected by the addition of 2-mercaptoethanol (Table 6). The enzyme activity was inhibited by sodium cyanide. As shown in Figure 6, the inhibition was noncompetitive with acrylonitrile as substrate (Km 0.21 *M*), the Ki value of sodium cyanide was 0.13 m*M*.

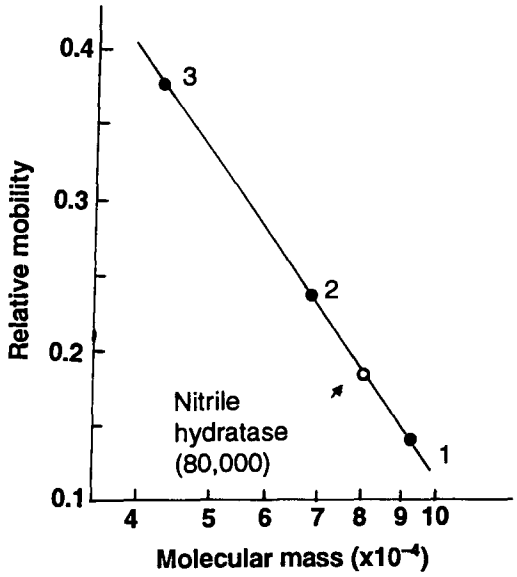

Fig. 4. Determination of molecular mass of nitrile hydratase by gradient PAGE. The molecular mass of reference proteins are: 1. Phosphorylase b, 94,000; 2. Bovine serum albumin, 68,000; 3. Ovalbumin, 43,000.

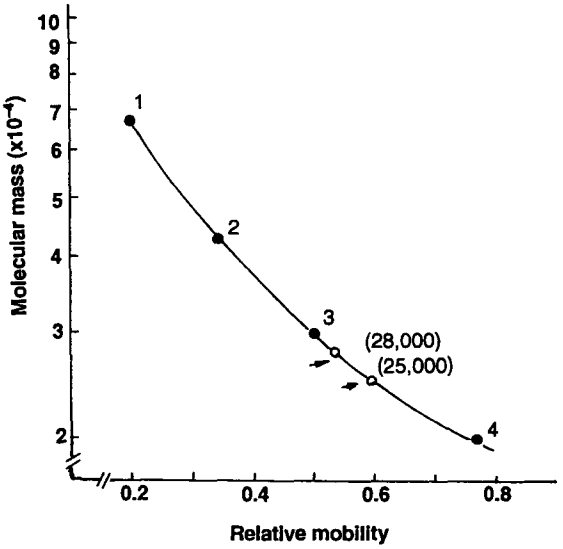

Fig. 5. Determination of molecular mass of subunits by SDS-PAGE. The molecular mass of reference proteins are: 1. Bovine serum albumin, 68,000; 2. Ovalbumin, 43,000; 3. Carbonic anhydrase, 30,000; 4. Trypsin inhibitor, 20,100.

Table 5
Effect of Metal Ions and Inhibitors on Activity of Nitrile Hydratase

Reagents	Concentration, m*M*	Relative activity, %
None		100
$AgNO_3$	0.1	0
$HgCl_2$	0.1	0
$CuCl_2$	1.0	26
Iodoacetate	1.0	52
Pheyl mercuric acetate	1.0	0
EDTA	1.0	30

Table 6
Protection of 2-Mercaptoethanol on Activity of Nitrile Hydratase

Inhibitors	Concentration, m*M*	Relative activity, %	
		2-Mercaptoethanol, 4 m*M*	
		None	Added
None		100	95
$HgCl_2$	0.01	24	100
	0.10	0	88
Iodoacetate	1.0	17	99

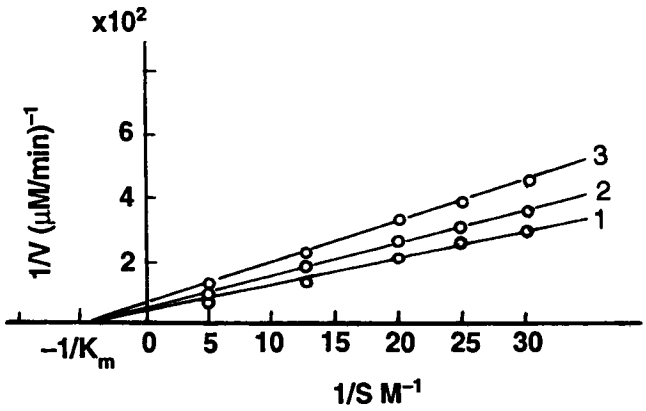

Fig. 6. Inhibition of nitrile hydratase by sodium cyanide. The concentrations of sodium cyanide are: 1. 0.0; 2. 0.005; 3. 0.01 m*M*.

The nitrile hydratase is an important catalyst for the production of acrylamide on an industrial scale, a very useful chemical commodity, by biotechnology. The formation and reaction conditions for nitrile hydratase from *C. pseudodiphteriticum* ZBB-41 that was isolated as a butyronitrile-utilizing strain have been established. The optimum temperature of the enzyme reaction was 25°C. This would be a promising method for acrylamide production as compared with the chemical hydration of acrylonitrile with respect to the specificity, reaction temperature, and ease of preparation of the enzyme. Therefore, the study on the enzyme formation and properties is necessary to the increase of yield and purity of acrylamide produced using bioconversion process.

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