Eur. J. Biochem. **263**, 662–670 (1999) © FEBS 1999

Nitrile hydratase involved in aldoxime metabolism from *Rhodococcus* sp. strain YH3-3

Purification and characterization

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Nitrile hydratase responsible for aldoxime metabolism from the *E*-pyridine-3-aldoxime degrading bacterium, *Rhodococcus* sp. strain YH3-3 was purified and characterized. Addition of cobalt ion was necessary for the formation of enzyme. The enzyme activity was highly induced not only by nitriles and amides but also by several aldoxime compounds. The enzyme was purified ≈ 108-fold with a 16% yield from the cell-free extract of the strain. The native enzyme had a M_r of ≈ 130 000 and consisted of two subunits (α-subunit, 27 100; β-subunit, 34 500). The enzyme contained approximately 2 mol cobalt per mol enzyme; it showed a maximum activity at 60 °C and at 40 °C under the rate assay and end-point assay conditions, respectively, and was stable over a wide range of pH (pH 2.5–11.0). The enzyme had a wide substrate specificity: it acted on aliphatic saturated and unsaturated as well as aromatic nitriles. The N-terminus of the β-subunit showed good sequence similarities with those of other nitrile hydratases. Nitrile hydratase is part of the metabolic pathway for aldoximes in microorganisms.

Keywords: aldoxime; nitrile hydratase; nitrile; oxime metabolism; *Rhodococcus* sp.

Nitrile compounds are used extensively in the chemical industry. They are discharged into the environment in industrial waste water, agricultural chemicals, etc. We have studied the microbial degradation of nitrile compounds and isolated various nitrile-degrading microorganisms from soil, such as *Rhodococcus rhodochrous* (formerly *Arthrobacter* sp.) J-1 and I-9 [1], *Pseudomonas chlororaphis* B23 [2], and a *Fusarium* sp. [3]. It was clarified that nitriles are converted to amides by nitrile hydratase [4] and to carboxylic acids by nitrilase [5] or by a combination of nitrile hydratase and amidase [6]. Nitrile hydratase was first purified and characterized from the cells of *R. rhodochrous* J-1 [4,7]. This pioneering research attracted much attention and the enzyme has subsequently been purified and characterized from *Rhodococcus* [8–12], *Pseudomonas* [13], *Pseudonocardia* [14], and *Bacillus* [15]. Recently, the three-dimensional structure of iron-containing nitrile hydratase from *Rhodococcus* sp. N-774 and R-312 was reported [16,17]. However, the reaction mechanisms of the enzyme are still not well understood. We demonstrated that *Ps. chlororaphis* B23 accumulates a large amount of amides from nitriles and is suitable for the industrial production of acrylamide from acrylonitrile [2,18]. The industrial production of 5-cyanovaleramide from adiponitrile has begun also [19]. Despite the importance of the enzymes for industrial use, there had been no report concerning the biosynthesis of nitriles and physiological function as the enzymes were screened only from nitrile-degrading microorganisms.

We have been studying aldoxime-degrading enzyme, not only to clarify the microbial metabolism of aldoxime but also to apply the enzyme to organic synthesis. We screened for various aldoxime-degrading microorganisms from soil and isolated a bacterium, *Rhodococcus* sp. strain YH3-3, capable of utilizing (*E*)-pyridine-3-aldoxime as a nitrogen source, after a 4-month acclimatization [20] and used it for nitrile synthesis under mild conditions [21]. The strain had a novel aldoxime dehydratase that catalyses a stoichiometric dehydration of (*E*)-pyridine-3-aldoxime to form 3-cyanopyridine (3-CP); the enzyme was induced by various aldoximes and nitriles. The strain metabolized the aldoxime as follows: (*E*)-pyridine-3-aldoxime was dehydrated to form 3-CP, which was converted into nicotinamide with a nitrile hydratase, and the nicotinamide was successively hydrolysed to nicotinic acid by an amidase [20]. To elucidate a possible relationship between aldoxime dehydratase and nitrile degrading enzymes and to clarify the reaction mechanisms of the enzyme, here we report the purification and characterization of nitrile hydratase involved in aldoxime metabolism from the strain and compare its enzyme properties with those of nitrile hydratases from various microorganisms.

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Abbreviations: 3-CP, 3-cyanopyridine; NA, nicotinamide; KPB, potassium phosphate buffer.

Enzymes: nitrile hydratase (EC 4.2.1.29); aldoxime dehydratase (EC 4.2.1.-).

(Received 8 March 1999, accepted 5 May 1999)

MATERIALS AND METHODS

Materials

Polypeptone and yeast extract were from Nippon Seiyaku (Osaka, Japan); meat extract was from Kyokuto (Tokyo, Japan); NZ-AMINE™, NZ-CASE™, and corn steep liquor were from Wako Pure Chemicals (Osaka, Japan). HPLC columns, ODS-80Ts, Octyl-80Ts, and G-3000SW, DEAE-Toyopearl, and Butyl-Toyopearl were from Tosoh (Tokyo, Japan). Gigapite was from Seikagaku Kogyo (Tokyo, Japan). Hibar Lichrosorb-NH_2

column was from Kanto Chemicals (Tokyo, Japan). Columns for Bio-Pilot systems, Q-Sepharose 35/100, Phenyl-Sepharose 35/100, and Superdex^{TM} 200 35/600 were from Pharmacia. Aldoxime compounds were synthesized from the corresponding aldehydes with hydroxylamine as described previously [20,21]. All other chemicals were from commercial sources.

Microorganism, media and growth conditions

Rhodococcus sp. strain YH 3-3, isolated from soil [20], was used throughout the study. A nutrient medium consisting of 10 g·L^{-1} polypeptone, 10 g·L^{-1} meat extract and 5 g·L^{-1} NaCl in tap water (pH 7.2) was used. Basal medium for the optimization of culture condition consisted of 0.2% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4{\cdot}7H_2O$, 0.05% yeast extract, and 1% vitamin mixture (pH 8.0) [17]. One colony of the strain grown on the nutrient plate was inoculated into a test tube (1.4 × 18 cm) containing 5 mL of the nutrient broth and cultivated at 30 °C for 48 h with reciprocal shaking at 310 r.p.m. Five hundred microlitres of the culture was inoculated into 10 mL basal medium containing various carbon and nitrogen sources, and inducers in a test tube (2.6 × 20 cm) and incubated at 30 °C for 48 h with shaking at 310 r.p.m.

The cell growth of the strain was estimated turbidimetrically by means of a dry cell weight calibration by the absorbance at 610 nm with a Hitachi U-3210 spectrophotometer (Hitachi, Japan); 0.75 mg dry cell weight per mL was found to be equivalent to an A_{610} of 1.0 unit.

Nitrile hydratase assay

One unit of the enzyme activity was defined as the amount of enzyme that produced 1 μmol amide from nitrile per min under the following assay conditions.

End-point assay. The enzyme activity was assayed by measuring the production of nicotinamide (NA) from 3-CP in a reaction mixture (500 μL) containing 2.5 mg sodium valerate, 50 μmol Hepes buffer (pH 7.2), 25 μmol CP and an appropriate amount of cells or enzymes. The reaction was performed at 30 °C for 10 min and stopped by adding 4.5 mL 50 mM KH_2PO_4/H_3PO_4 buffer (pH 2.8) in 50% 2-propanol. The amount of NA formed in the reaction mixture was determined by HPLC with a Hibar Lichrosorb-NH_2 column at 254 nm with a mobile phase of 75% CH_3CN containing 10 mM KH_2PO_4/H_3PO_4 (pH 2.8) at a flow rate of 1.0 mL·min^{-1}.

Rate assay. The enzyme activity was assayed at 30 °C as the rate of formation of methacrylamide from 10 mM methacrylonitrile in 0.1 M potassium phosphate buffer (KPB, pH 7.0) containing 0.5% sodium valerate at pH 7.0 by measuring absorbance at 224 nm ($\Delta\varepsilon$ = 3400 M^{-1} cm^{-1}).

Purification of nitrile hydratase from *Rhodococcus* sp. strain YH3-3

The strain was grown in basal medium containing 1% sodium pyruvate, 0.2% NZ-AMINE^{TM}, 0.15% isovaleramide and 0.001% $CoCl_2$ at 30 °C for 48 h. Unless otherwise stated, all purification procedures were performed at 4 °C. Hepes buffer (pH 7.2) or KPB (pH 7.0) containing 0.5% sodium valerate was used throughout the purification.

Step 1. Preparation of cell free extract. The washed cells (253 g-wet weight from 48 L of culture) were suspended in 4 L 100 mM Hepes buffer. The cells were disrupted four times with 0.25–0.50 mm diameter glass beads (DYNO-MILL KDL; W. A. Bachofen, Switzerland) and the disrupted cells were removed by centrifugation at 18 800 ***g*** for 15 min.

Step 2. $(NH_4)_2SO_4$ precipitation. The cell-free extract was fractionated with $(NH_4)_2SO_4$. Proteins precipitating between 30 and 60% saturation were collected by centrifugation (18 800 ***g***, 20 min), dissolved in a small amount of the 10 mM Hepes buffer and dialysed against the same buffer.

Step 3. Butyl-Toyopearl column chromatography. The enzyme solution, to which solid $(NH_4)_2SO_4$ has been added to 30% saturation, was placed on a Butyl-Toyopearl 650M column (4 × 30 cm) which had been equilibrated with 10 mM Hepes buffer containing 30% saturated $(NH_4)_2SO_4$. After the column had been washed with the same buffer, the enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$ (30–0% saturation, 1 L each).

Step 4. DEAE-Toyopearl column chromatography. The active enzyme solution was dialysed against 10 mM KPB and applied to a DEAE-Toyopearl 650M column (4 × 24 cm) which had been equilibrated with 10 mM Hepes buffer. After the column had been washed with 100 mM Hepes buffer, the enzyme was eluted with a linear gradient of NaCl (0–500 mM, 500 mL each) in the same buffer. The active fractions were collected and dialysed against 10 mM KPB.

Step 5. Gigapite column chromatography. The dialysed enzyme solution was applied to a Gigapite column (5 × 25 cm) equilibrated with 10 mM KPB. After the column had been washed with 10 mM KPB, the enzyme was eluted with a linear gradient of KPB (10–250 mM, 750 mL each).

Step 6. Q-Sepharose 35/100 column chromatography. At steps 6, 7, and 8, the Bio-Pilot System (Pharmacia) was used at a flow rate of 10 mL·min^{-1}. The active enzyme solution, which had been dialysed against 10 mM KPB, was loaded on a Q-Sepharose 35/100 column equilibrated with 20 mM KPB. After the column had been washed with 500 mL of the same buffer, the enzyme was eluted with a linear gradient of NaCl (200–300 mM, 1 L each) in the same buffer and the active fractions were collected.

Step 7. Phenyl-Sepharose 35/100 column chromatography. The dialysed enzyme solution, to which $(NH_4)_2SO_4$ had been added to 30% saturation, was applied to a Phenyl-Sepharose 35/100 column equilibrated with 10 mM KPB containing 30% saturated $(NH_4)_2SO_4$. After the column had been washed with 500 mL of the same buffer, the enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$ (30–0%, 500 mL each) in 10 mM KPB.

Step. 8. Superdex 200^{TM} 35/600 column chromatography. The dialysed active enzyme solution was placed on a Superdex 200^{TM} 35/600 column equilibrated with 50 mM KPB containing 100 mM NaCl and eluted with 600 mL of the same buffer.

Analytical methods

Protein concentrations were determined with a Bio-Rad protein assay kit [22] using BSA as standard or by measuring absorbance at 280 nm. Native and SDS/PAGE were carried out as described by Davis [23] and Laemmli [24], respectively, with an electrophoresis unit model by Atto (Tokyo, Japan). The

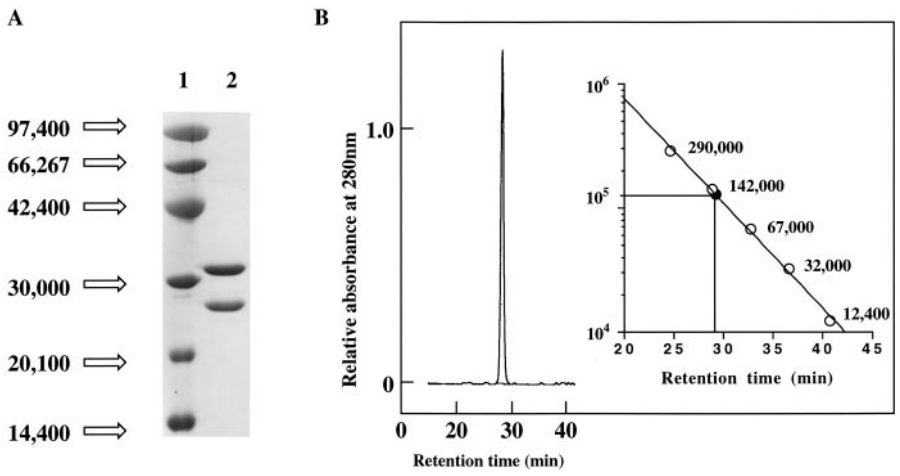

Fig. 1. Analysis of purified nitrile hydratase. (A) SDS/PAGE. Lane 1, standards: phosphorylase (M_r 97 400), BSA (M_r 66 267), aldolase (M_r 42 400), carbonic anhydrase (M_r 30 000) and soybean trypsin inhibitor (M_r 20 000). Lane 2, purified nitrile hydratase (10 μg). (B) High performance gel filtration chromatography: the elution profile of the enzyme (●) and M_r of the enzyme are shown. The protein standards (○), in order of decreasing M_r, were glutamate dehydrogenase (M_r 290 000), lactate dehydrogenase (M_r 142 000), enolase (M_r 67 000), adenylate kinase (M_r 32 000), and cytochrome *c* (M_r 12 400). The UV absorption at 280 nm was expressed as the relative absorbance which represents the percentage of full-scale deflection on the recorder expressed from 0 to 1.0 on the ordinate.

relative molecular mass (M_r) of the enzyme was estimated as described previously [25]. For an analysis of the cobalt content of the enzyme, the enzyme sample (0.102 mg·mL^{-1}) was measured with an atomic absorption flame emission spectrophotometer (Nippon Jarrell-Ash, Kyoto, Japan).

N-terminal amino acid sequence analysis

The subunits of the enzyme were separated by HPLC using an Octyl-80Ts column (4.6 × 150 mm) at 280 nm. The enzyme (100 μg) was incubated in 100 μL of 40% CH_3CN containing 0.1% of trifluoroacetic acid at 30 °C for 1 h. Separation was achieved with an increasing linear gradient of CH_3CN (10–80%) containing 0.1% trifluoroacetic acid at a flow rate of 0.5 mL·min^{-1}. The concentrated enzyme solution was covalently bound to Sequelone-diisothiocyanate and Sequelone-arylamine membranes and then analysed with a Prosequencer 6625 automatic protein sequencer (Millipore, USA). BLAST and FASTA searches were performed to detect similarities between the N-terminal amino acid sequence of the enzyme and other known proteins.

RESULTS

Effects of cultivation conditions on the formation of nitrile hydratase

As the production of iron- and cobalt-containing nitrile hydratases has been known to be enhanced by the addition of the corresponding metal ions to the cultivation medium [8,26], we investigated the effects of various metal ions on the enzyme formation in *Rhodococcus* sp. strain YH 3-3. As shown in Table 1, addition of cobalt ions was required for the formation of nitrile hydratase in cells of the strain. The total and specific activity in the cells grown with $CoCl_2$ was about 100- and 1200-fold, respectively, than those in the cells grown without metal ions. The optimum $CoCl_2$ concentration was estimated to be 0.001–0.005%. Very low (< 0.3 U·L^{-1}) activity was seen in the strain grown in the medium containing the following metal ions; Li^+, Mg^{2+}, Al^{3+}, K^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Ni^{2+}, Rb^+, Cd^{2+}, Sn^{2+}, Ba^{2+}, and Pb^{2+}.

The effect of inducers on the formation of the enzyme activity was examined. As shown in Table 2, the enzyme activity was induced by various amides, nitriles, and aldoximes, especially isovaleramide, (*E/Z*)-isovaleraldoxime, methacrylamide, crotononitrile, and crotonamide. Conversely, (*E*)-*N*-methyl-pyridine-3-aldoxime, 3-CP-*N*-oxide, (*E*)-*O*-methyl-pyridine-3-aldoxime, (*E/Z*)-capronaldoxime, (*E/Z*)-isocapronaldoxime, acetamide, and urea inhibited cell growth. Among the inducers tested, isovaleramide was selected as the best one for the enzyme formation, although the enzyme of *R. rhodochrous* J1 was induced by ε-caprolactam and urea [26].

Supplementation with various nitrogen and carbon sources was investigated. Among the nitrogen sources tested, NZ-AMINE™ supported the cell growth most without decreasing specific activity. The strain could grow neither on inorganic sources of nitrogen such as $(NH_4)_2SO_4$, $NaNO_3$, $NaNO_2$, NH_4Cl, or $(NH_4)_2HPO_4$, nor on organic sources such as malt extract or urea. Organic acids (e.g. pyruvate and lactate) and sugars (e.g. starch and gluconate) were effective in promoting cell growth and the enzyme activity, whereas other organic acids and sugars did not enhance the enzyme activity although the cells grew well in their presence.

Based on these results, we have optimized the culture conditions for the enzyme formation as follows: the strain was grown aerobically for 50 h in basal medium containing 0.15% isovaleramide, 0.2% NZ-AMINE™ and 1.0% sodium pyruvate as inducer, nitrogen and carbon sources, respectively, and with 0.001% $CoCl_2$.

Table 1. Effect of various metal ions on the formation of nitrile hydratase in *Rhodococcus* sp. YH 3-3. The strain was cultivated at 30 °C for 48 h in a test tube containing 10 mL of basal medium with 1% of sodium acetate, 0.2% NZ-AMINE™, 0.15% crotononitrile and 0.001% (w/v) various metal ions. The enzyme activity was measured under the end-point assay conditions.

Metal ion	Cell growth (g dry cell·L culture^{-1})	Total activity (U·L culture^{-1})	Specific activity (U·g dry cell^{-1})
$CoCl_2$	5.81	485	83.5
$[Co(NH_3)_6]Cl_3$	6.06	470	77.6
$FeSO_4$	5.16	4.96	0.961
$ZnCl_2$	5.55	1.26	0.227
$FeCl_3$	7.32	0.99	0.135
$CuSO_4$	6.34	0.66	0.104
None	5.75	0.41	0.071

Table 2. Effect of various inducers on the formation of nitrile hydratase. Various inducers (0.15%) were added to basal medium containing 1% sodium acetate, 0.2% NZ-AMINE^{TM}, and 0.001% $CoCl_2$. Cultivation was carried out for 48 h at 30 °C with shaking. The enzyme activity was measured under the end-point assay conditions.

Inducer	Growth (g dry cell·L culture^{-1})	Total activity (U·L culture^{-1})	Specific activity (U·g dry cell^{-1})
Isovaleramide	6.23	2780	446
Crotonamide	4.17	2280	547
(*E*/*Z*)-Isovaleraldoxime	5.46	2180	399
Methacrylamide	4.74	2070	437;
Crotononitrile	5.88	1970	335
Furfurylamide	4.95	1880	380
Isovaleronitrile	4.94	1570	318
Propionamide	5.62	1420	253
Methacryronitrile	4.91	1040	212
Propionitrile	5.45	1020	187
(*E*/*Z*)-*n*-Butyraldoxime	5.65	992	176
n-Valeronitrile	6.28	983	157
Nicotinamide	4.90	832	170
n-Valeramide	6.05	706	117
n-Butyramide	5.47	612	112
(*E*/*Z*)-Propionaldoxime	4.37	582	133
n-Butyronitrile	4.84	430	88.8
n-Capronamide	6.35	298	46.9
(*E*/*Z*)-Isobutyraldoxime	4.03	297	73.7
Phenylacetamide	6.23	260	41.8
2-Picolinamide	5.36	238	44.4
Isobutyramide	5.02	225	44.8
n-Capronitrile	0.653	111	170
None	0.738	9.19	1.24

Purification of nitrile hydratase from *Rhodococcus* sp. strain YH3-3

No nitrile hydratase activity was detected in the cell-free extract after disruption of the cells in several buffers at various pH values suggesting that the enzyme was very labile after extraction from the cells. It was reported that several organic acids could protect nitrile hydratase from inactivation during extraction [9,12]. The enzyme of this study could be stabilized also by organic acids such as butyrate, valerate and caproate; therefore, 0.5% sodium valerate was added to the buffers throughout the purification procedure. As summarized in Table 3, the enzyme was purified to homogeneity 108-fold with an overall yield of 16% from the cell-free extract of the strain, by $(NH_4)_2SO_4$ fractionation, and ion-exchange, hydrophobic and gel-filtration column chromatographies. The enzyme was judged to be homogeneous by native-PAGE

Table 3. Summary of purification of nitrile hydratase from *Rhodococcus* sp. strain YH 3-3. The enzyme activity was measured under the end-point assay conditions.

Step	Total protein (mg)	Total activity (U)	Specific activity (U·mg^{-1})	Yield (%)
Cell-free extract	61 100	67 900	1.11	100
$(NH_4)_2SO_4$ fractionation	43 300	29 900	1.45	44.0
Butyl-Toyopearl	2560	36 000	14.1	53.0
DEAE-Toyopearl	794	35 600	44.8	52.4
Gigapite	366	34 300	93.7	50.5
Q-Sepharose 35/100	280	32 000	114	47.1
Phenyl Sepharose 35/100	209	24 600	117	36.2
Superdex 200	90.7	10 900	120	16.0

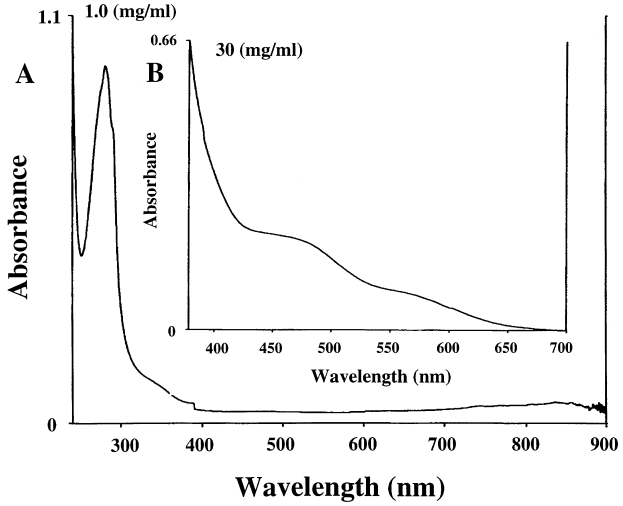

Fig. 2. Absorption spectrum of nitrile hydratase from *Rhodococcus* sp. strain YH3-3. The absorption spectrum of the enzyme was recorded using a Hitachi U-3210 spectrometer with the purified enzyme solution (A, 1.0 mg·mL^{-1}; B, 30 mg·mL^{-1}) in 10 mM Hepes buffer (pH 7.0) containing 0.5% valerate.

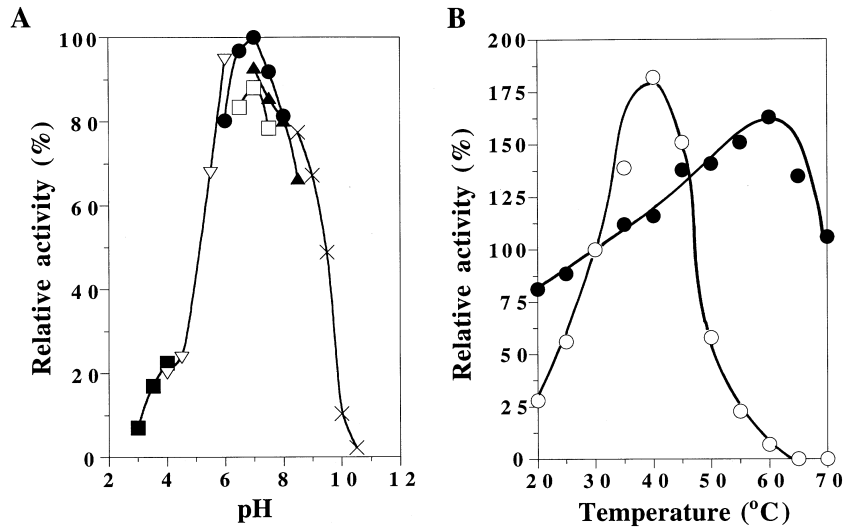

Fig. 3. The effect of pH and temperature on the activity of nitrile hydratase. (A) To assess the effect of pH the reactions were carried out under the rate assay conditions at 30 °C in the following buffers (100 mM): Gly-HCl (■), AcONa/AcOH (▽), KPB (●), Mops (□), Tris/HCl (X), and Gly-NaCl-NaOH (▲). (B) Effect of temperature: reactions were carried out under the rate assay (●) and the end-point assay (○) conditions at various temperatures. The activity is expressed relative to that at 30 °C in KPB (pH 7.0).

(data not shown) and HPLC on a G-3000SW column: each of these procedures yielded a single band or a single peak (Fig. 1).

M_r and subunit structure

As shown in Fig. 1, the native M_r of the enzyme was calculated to be about 130 000 by gel filtration on HPLC with a G-3000 SW column; the subunit M_r were estimated to be ≈34 500 (β-subunit) and 27 100 (α-subunit), respectively, by comparing the mobility on SDS/PAGE to that of standard proteins (Fig. 1). It was shown that the enzyme appears to exist in $\alpha_2\beta_2$ form. Ampholitic IEF with the Phast System (Pharmacia) yielded only one band and its pI value was 6.11.

Prosthetic group of the enzyme

A concentrated solution of the purified enzyme was faint pink, suggesting that the enzyme might contain some cofactors. Cobalt-containing nitrile hydratase from *R. rhodochrous* J1 [12] and iron-containing enzyme from *Rhodococcus* sp. R312 [8], N-774 [10], and *Ps. chlororaphis* B23 [11] showed an absorption maximum at around 410 nm and 710 nm, respectively. As shown in Fig. 2, the enzyme exhibited absorption in a broad region over 300–500 nm with a shoulder at around 470–500 nm. Quantitative analysis of metal ions from the enzyme solution with the atomic absorption spectrum revealed that the enzyme had two cobalt atoms per mol of enzyme. As the enzyme was suggested to have an $\alpha_2\beta_2$ structure, it may contain one cobalt atom per αβ subunit. These cobalt atoms were tightly bound to the protein and were not liberated by dialysis against KPB, with or without valerate, for 1 month.

No effect was seen on the enzyme activity with the following coenzymes: phenazinemethosulfate (PMS), FAD, NAD^+, NADH, $NADP^+$, NADPH, thiooctic acid, pyridoxal 5′-phosphate, ATP, ADP, AMP, IMP, GMP, IDP, UDP, thioglycolic

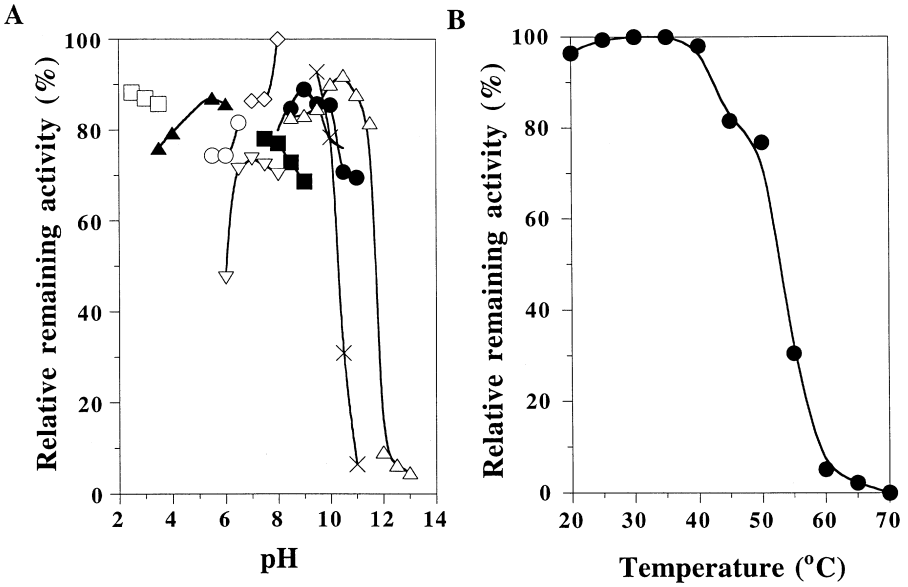

Fig. 4. The effect of pH and temperature on the stability of nitrile hydratase. (A) To assess the effect of pH enzyme activity was measured under the rate assay conditions after preincubation at 30 °C for 30 min in the following buffers (100 mM): Gly-HCl (□), AcONa/AcOH (▲), KPB (▽), Hepes (◇), Mes (○), Tris/HCl (■), ethanolamine/HCl (●), $NaHCO_3$-Na_2CO_3 (X), and Gly-NaCl-NaOH (△). (B) Effect of temperature: activity was measured after preincubation for 30 min at various temperatures in 50 mM KPB (pH 7.0). The activity remaining is expressed relative to that of untreated enzyme.

Fig. 5. N-terminal amino acid sequence homology of nitrile hydratase. YH3-3, *Rhodococcus* sp. strain YH3-3 (this study); J1-High, *R. rhodochrous* J1 high-MW [28]; J1-Low, *R. rhodochrous* J1 low-MW [28]; NRRL-18668, *Ps. putida* NRRL-18668 [13]; M8, *Rhodococcus* sp. M8 [29]; R312, N-774, *Rhodococcus* sp. R312 (N-771, 774) [30]; B23, *Ps. chlororaphis* B23 [31]; *Rhodococcus* sp., *Rhodococcus* sp. [32]; MCI 2609, *Klebsiella* sp. MCI 2609 [33]; IFO 12668, *Achromobacter xerosis* IFO 12668 [34]; N1, *Commamonas testosteroni* N1 [35]; MCI 2643, *Rhizobium* sp. MCI 2643 [36]; JCM 3095, *Pseudonocardia thermophila* JCM 3095 [14]; RAPc8, *Bacillus* sp. RAPc8 [15].

acid, cysteamine, glutathione (oxidized and reduced form), L-cysteine, iodonitrotetrazolium, dehydroascorbic acid, and L-ascorbic acid.

Effect of temperature and pH on the enzyme activity

The effect of temperature and pH on the enzyme activity was examined. The activity was measured under the rate assay conditions at various pH values in several buffers at 30 °C. The enzyme showed maximum activity at pH ≈ 7.0 in KPB (Fig. 3A). The enzyme reaction was carried out at various temperatures in KPB (pH 7.0). As shown in Fig. 3B, the enzyme showed a maximum activity at 60 °C and at 40 °C under the rate assay and end-point assay conditions, respectively.

Effect of temperature and pH on the enzyme stability

The enzyme was preincubated for 30 min in 100 mM buffer at various pH values at 30 °C; an aliquot of each enzyme solution was then taken and the enzyme activity was assayed. As shown in Fig. 4A, the enzyme was stable between pH 2.5 and pH 11.0. The remaining activity of the enzyme was measured after an incubation in 50 mM KPB (pH 7.0) at various temperatures. The enzyme was stable up to 40 °C and its activity was inhibited above 50 °C (Fig. 4B).

Effect of various compounds on the activity of the enzyme

The enzyme was incubated at 30 °C for 30 min with various concentrations of metal ions and inhibitors and the enzyme activity was measured under the rate assay conditions. The enzyme activity was inhibited by heavy metal ions and carbonyl reagents. The enzyme activity was completely lost by incubating with 1 mM of $AgNO_3$ and $HgCl_2$. After incubation of the enzyme with phenylhydrazine (1 mM), KCN (10 mM), and NH_2OH (10 mM), only 5.88, 12.1, and 47.7% of the original enzyme activity remained. The activity was not inhibited by other sulfhydryl reagents, serine inhibitors, carbonyl reagents or other monovalent, divalent, and trivalent metal ions.

Inhibitory effects of sodium valerate on the methacrylonitrile hydration activity

As already mentioned, the enzyme required valerate for its stability during extraction from the cells. It was found that the added valerate inhibited the hydration reaction of methacrylonitrile to methacrylamide but not that of 3-CP to nicotinamide. To clarify the inhibition mechanism, the initial velocity v for the hydration of various concentrations, [S], of methacrylonitrile was

Table 4. Substrate specificity of nitrile hydratase. The enzyme activity for methacrylonitrile and acrylonitrile was measured in the reaction mixture (0.5 mL) with 20–200 pg of the enzyme under the rate assay conditions. The enzyme activity for other nitriles was assayed with 40 pg–10 μg enzyme·mL^{-1} reaction mixture under the end-point assay conditions. Aliphatic nitriles and amides were analysed by GLC (Shimadzu, Kyoto, Japan) using a flame ionization detector with a glass column (2 mm × 0.5 m) packed with Porapak-Q (80–100 mesh; Waters, USA). When cyanopyridines and cyanopyrazine were used as substrate, the amount of the corresponding amides formed was determined by HPLC with a Hibar Lichrosorb-NH_2 column and a solvent of 75% CH_3CN containing 10 mM KH_2PO_4/H_3PO_4 (pH 2.8) at a flow rate of 1.0 mL·min^{-1}. Other aromatic nitriles and amides were detected with a ODS-80Ts column using aqueous CH_3CN containing 10 mM H_3PO_4.

Substrate	Relative activity (%)[a]	K_m (mM)	V_{max} (U·mg^{-1})	V_{max}/K_m (U·mg^{-1}·mM^{-1})
3-CP	100	3.90	421	108
2-CP	376	2.24	7110	3170
4-CP	133	4.56	602	132
Cyanopyrazine	100	5.84	361	61.8
2-Cyanothiophene	276	0.982	856	872
2-Cyanofuran	479	0.578	478	827
Benzonitrile	226	0.145	354	2440
p-Tolunitrile	85.4	0.170	70.5	425
p-Chlorobenzonitrile	85.4	0.170	70.8	417
p-Nitrobenzonitrile	77.6	0.822	104	127
Phenylacetonitrile	247	6.01	2400	400
Propionitrile	278	77.8	1280	16.5
n-Butyronitrile	449	32.3	1220	37.8
Isobutyronitrile	56.3	5.33	13.2	2.48
n-Valeronitrile	654	5.02	902	180
Acrylonitrile	156	0.845	330	390
Crotononitrile	81.4	21.5	14.1	1.52
Methacrylonitrile	322	0.282	287	1020

[a] Measured at 10 mM concentration. The relative activity for acetonitrile, cinnamonitrile, 2-phenylpropionitrile and 3-phenylpropionitrile, 4-phenylbutyronitrile, mandelonitrile, and indolacetonitrile was 30.5, 0.412, 0.917, 0.171, 0.121, 0.235, and 0.271%, respectively.

Table 5. Properties of nitrile hydratases from various microorganisms.

Property	*Rhodococcus rhodochrous* J-1	*Pseudomonas chlororaphis* B-23	*Rhodococcus* sp. N-774 (R312)	*Rhodococcus rhodochrous* J1 (High-M_r)	*Pseudomonas putida* NRRL-18668	*Bacillus* sp. RAPc8	*Rhodococcus* sp. YH3-3
M_r (α)	24 000	22 117	22 865	22 834	22 982	28 000	27 100
(β)	27 000	24 545	23 487	26 321	24 108	29 000	34 500
Subunit structure	$(\alpha\beta)_n$	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha_{10}\beta_{10}$	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha_2\beta_2$
Stability pH	6.0–7.0	6.5–7.5	6.0–8.5	6.0–8.0	N.D.	N.D.	2.5–11.0
temperature (°C)	35	20	20	50	35	50	40
Optimum pH	7.0–7.2	7.5	7.8	6.5–6.8	7.2–7.8	7.0	7.0
temperature (°C)	35[a]	20[a]	25[a]	35[a]	40[b]	60[a]	40[a], 60[b]
Inhibitor	Heavy metal KCN Iodoacetic acid	Heavy metal Phenylhydrazine	Heavy metal SH-reagent	Heavy metal KCN	ND ND	Heavy metal SH-reagent	Heavy metal Phenylhydrazine
		*p*CMB[c], Tiron[d]					
Substrate	Alkylnitrile	Alkylnitrile	Alkylnitrile	Alkylnitrile Arylnitrile	Alkylnitrile Arylacetonitrile	Alkylnitrile	Alkylnitrile Arylnitrile
Prosthetic group	–	Fe	Fe	Co	Co	(Fe)	Co

[a] Determined by end-point assay; [b] determined by rate assay; [c] *p*-chloromercuribenzoic acid; and [d] 1,2-dihydroxybenzene-3,5-disulfonic acid.

studied in the presence of valerate (0–1 mM). On the double reciprocal plots of v against [S], all lines intersected on the $1/v$ axis, which meant that the enzyme was inhibited competitively. A K_i value of 0.273 mM was deduced from the secondary plot of the slope of each reciprocal line versus the inhibitor concentration (Dixon plots).

Substrate specificity and kinetic properties of the nitrile hydratase

Substrate specificity of the enzyme was examined and kinetic values for each substrate were determined from Hanes–Woolf plots (Table 4). The enzyme acted on several aryl-, alkyl-, and arylalkyl-nitriles. The highest relative activity was seen when *n*-valeronitrile was used as a substrate. The lowest K_m and the highest V_{max} values were for benzonitrile and 2-CP, respectively. The enzyme did not act on nitriles such as 2-quinolinecarbonitrile, *m*-phtalodinitrile, *N*-methyl-3-CP, 3-CP-*N*-oxide, *p*-methylphenylacetonitrile, *p*-methoxyphenylacetonitrile, *p*-hydroxyphenylacetonitrile, *p*-chlorophenylacetonitrile, diphenylacetonitrile, and thiophene-2-acetonitrile.

N-terminal amino acid sequence

Each subunit of the enzyme was separated by HPLC and the N-terminal amino acid sequences of the α and β subunits were determined to be MNISTTAACTTRVVAALSMLLISGLVVTT and MNGLFDLGGVDGLGPINASCQEPAFHAPWECVVLTLFP respectively. As shown in Fig. 5, prominent similarities in the N-terminal amino acid sequences to other nitrile hydratases were observed for the β-subunit. The α-subunit, however, was less similar to other nitrile hydratases.

DISCUSSION

Here we describe the purification and characterization of nitrile hydratase which is involved in aldoxime metabolism from the (*E*)-pyridine-3-aldoxime degrading bacterium, *Rhodococcus* sp. strain YH3-3. The nitrile hydratase of this strain was similarly induced by cobalt ion as has been shown in *R. rhodochrous* J1 [27], in contrast with the nitrile hydratase of *P. chlororaphis* B23 and *Rhodococcus* R-312 [26], which are induced by iron ion. We discovered that nitrile hydratase was strongly induced not only by amides and nitriles but also by various aldoximes in the strain. To clarify the induction mechanisms, the strain was grown in medium containing (*E*/*Z*)-isovaleraldoxime and the aldoxime concentrations were measured by GLC during cultivation. No remarkable decrease in the aldoxime concentration was seen during the cultivation (data not shown). Thus, the enzyme was induced by aldoxime but not by its metabolites, such as isovaleronitrile, isovaleramide, and isovalerate.

The purified enzyme (native M_r 130 000) was composed of two subunits (α subunit, M_r 27 100; β subunit, M_r 34 500) and contained one cobalt molecule per αβ unit. In M_r and subunit composition the enzyme is similar to other nitrile hydratases, but not to the high-M_r nitrile hydratase of *R. rhodochrous* J1 which is composed of about $(\alpha\beta)_{10}$ [12]. Table 5 shows a comparison of the properties of this enzyme with nitrile hydratases from various microorganisms. The enzyme showed excellent pH stability and exhibited maximum activity at high temperature compared with known nitrile hydratases except the nitrile hydratase from the thermophilic bacterium, *Bacillus* sp. RAPc8, the optimum temperature of which was around 60 °C under the rate assay conditions [15].

The N-terminal sequences of the β-subunit of the enzyme were similar to those of other nitrile hydratases but those of the α-subunits were not. The α-subunits would be less homologous because this region is not functionally important. Recently, the three-dimensional structure of iron-containing nitrile hydratase from *Rhodococcus* sp. R-312 and N-774 was elucidated by X-ray crystallographic analysis [16,17]. The N-terminal part of the β-subunit was buried in the centre of the $\alpha_2\beta_2$ heterotetramer interface whereas that of the α-subunit was not although it is involved in stabilization of the heterotetramer (it wraps across the surface of the iron-containing enzyme). Furthermore, the N-terminus of the β-subunit was near to the active site in the iron-containing enzyme. Structural comparisons between cobalt-containing nitrile hydratases and iron-containing enzymes should reveal the similarity and evolutionary relationship between iron- and cobalt-containing nitrile hydratases.

It has been reported that nitrile hydratases are very labile to dilution and can be stabilized by organic acids such as butyrate

or valerate [9,11]. Iron-containing nitrile hydratase from *Rhodococcus* sp. R-312 liberated iron molecules on dialysis against buffer which did not contain organic acid [37]. Although the enzyme of the present study was also unstable on extraction from the cells and was stabilized by the addition of 0.5% of valerate, it did not loose cobalt ions when dialysed.

Iron-containing nitrile hydratase had a narrow substrate specificity, acting only on alkylnitriles [8–11], whereas its cobalt-containing counterparts widely acted on alkyl-, aryl-, and arylalkyl-nitriles [12,13]. The enzyme in this study, which contained cobalt ion, also had a wide substrate specificity and acted on saturated or unsaturated alkylnitriles as well as arylnitriles and arylalkylnitriles. The enzyme was found to be inhibited competitively by valerate when methacrylonitrile was used as a substrate; however, no inhibition was seen for the hydration reaction of 3-CP. These results suggest that the enzyme has two different substrate-binding pockets near the active centre: one binds to the alkyl group of alkylnitriles and the other binds to the aromatic ring of arylnitriles. Valerate should occupy the former pocket with its alkyl group, which inhibits the binding of the alkyl moiety of methacrylonitrile, resulting in the competitive inhibition of the reaction. Huang *et al*. revealed that the iron atom, accessible to the bulk solution, was bound at an open cavity that is very probably the active-site pocket of iron-containing nitrile hydratase by X-ray crystallographic analysis [16], but our experimental results do not agree with this observation. A comparison of the structures of iron- and cobalt-containing nitrile hydratases would elucidate the differences of substrate specificity between the enzymes.

In this study, we focused on a nitrile hydratase from an aldoxime-degrading microorganism. We found that the purified enzyme, which was highly induced by aldoxime compounds, showed similar enzymological properties to known nitrile hydratases. It was suggested that nitrile hydratase is part of the metabolic pathway for aldoximes in microorganisms. Molecular cloning of the aldoxime-degrading enzyme and nitrile hydratase genes of the strain should provide details as to the relationship between the enzymes at the gene level.

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