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Nitrile hydratase from a thermophilic *Bacillus smithii*

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A novel thermophilic *Bacillus smithii* strain SC-J05-1, isolated from a hot spring, had the ability of hydrating nitrile to form amide. The nitrile hydratase was purified to homogeneity from the microbial cells of SC-J05-1 and was characterized. The enzyme was a 130-kDa protein composed of two different subunits (25.3 kDa and 26.8 kDa) and contained cobalt ions. This enzyme had the optimal temperature of 40°C and was stable up to 50°C. The optimal pH was in the alkaline region higher than pH 10.

Keywords: nitrile hydratase; thermophile; *Bacillus smithii*

Introduction

Since the first discovery of the nitrile hydratase which converts nitriles to the corresponding amides was made with an *Arthrobacter* bacterium [1], several related enzymes have been found in microorganisms [4,6,9,10,13,15,17]. *Rhodococcus*, *Pseudomonas*, *Brevibacterium* are currently known as major nitrile hydratase-producing microorganisms, and the nitrile hydratases are regarded as participants in the metabolism of nitrile compounds. Many studies of these nitrile hydratases have been carried out, and some of them have been used industrially as biocatalysts for production of acrylamide (Figure 1) and nicotinamide [2,8,12].

From a practical point of view, the nitrile hydratases previously reported were not satisfactory catalysts because of their relatively low thermostability. Actually, the nitrile hydratase catalyzed reaction in the industrial production of acrylamide has been conducted at low temperature with cooling facilities in order to reduce the inactivation of the enzyme. The availability of a thermostable nitrile hydratase will help to solve this problem and will provide an expansion of the uses of the enzyme in the organic synthesis.

In this paper, we describe the discovery of *Bacillus smithii* strain SC-J05-1 having amide-forming ability, through the screening in our collection of moderate thermophiles isolated from soils, and studies on some characteristics of the nitrile hydratase purified from *B. smithii* SC-J05-1.

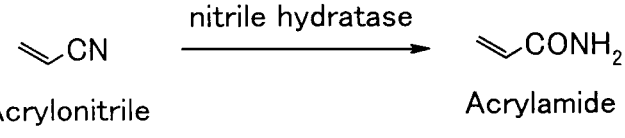

Figure 1 Transformation scheme of acrylonitrile to acrylamide catalyzed by nitrile hydratase.

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Materials and methods

Screening of thermophiles for amide-forming ability

The microorganisms used for the screening were our collection of moderate thermophiles that had been isolated from soil samples exposed at high temperature (40–80°C) through cultivation in a Bact Nutrient Broth at 55°C. The strains had been suspended in 30% glycerol aqueous solution and then stored at −80°C. Each stored strain was inoculated in nutrient medium consisting of (per L): 5 g polypeptone, 3 g yeast extract, 3 g malt extract, 10 g glycerol, and 2 g isovaleronitrile; and cultivated aerobically at 55°C for 2 days.

The cultured broth was subjected to measurement of the amide-forming ability which was carried out as follows: 0.5 ml of a cultured broth was added to 4.5 ml of 50 mM potassium phosphate buffer (pH 7.7) containing 2.36 mmol of acrylonitrile and incubated at 10°C for 10 min. The reaction was stopped by adding 0.5 ml of 2 N HCl to the reaction mixture. The amount of amide formed in the mixture was determined by gas chromatography with a GC-9A (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector. The column used was a glass column (2.6 mm i.d., 1.1 m length) packed with Porapak Q (80–100 mesh) (Millipore Corporation, Milford, MA, USA). The operational conditions were: column temperature, 210°C; injector and detector temperature, 240°C; and carrier gas, N_2 at a flow rate of 50 ml min^{-1}. The integration and calibration of peak area were carried out with a Chromatopac C-R6A (Shimadzu Corporation, Kyoto, Japan).

Assay of nitrile hydratase and definition of unit of the activity

An assay of the nitrile hydratase was done by measuring the initial reaction rate of hydration in the reaction mixture (5 ml) consisting of propionitrile (2.36 mmol) and enzyme solution (0.5 ml) in 50 mM potassium phosphate buffer (pH 7.7). When the activity was measured at various pHs, sodium citrate (pH 5.0–6.0), potassium phosphate (pH 6.0–8.0), Tris-HCl (pH 8.0–9.0), and glycine-HCl (pH 9.0–10.5) were used for the buffers. The reaction was carried out at 10°C for 10 min and then stopped by adding 0.5 ml of 2 N HCl to the reaction mixture. The amount of amide

Table 1 Taxonomical properties of strain SC-J05-1

(A) Morphology	1. Shape and size of cells	Shape: rods Size: 0.5–0.8 × 0.8–2.0 μm	
	2. Polymorphism: none		
	3. Motile: active (peritrichous flagella)		
	4. Sporulation: formed		
	5. Gram staining: variable		
	6. Acid fastness: none		
(B) Growth properties	1. Broth agar plate culture: round, convex colonies non glossy, pale brown		
	2. Broth agar slant culture: non glossy, pale brown		
	3. Broth liquid culture: uniform turbid growth		
	4. Broth gelatin stab culture: not liquefied		
	5. Litmus milk: nonreactable		
(C) Physiological properties	1. Nitrate reduction:	–	
	2. Denitrification:	–	
	3. Methyl red test:	+	
	4. Voges-Prokauer test:	–	
	5. Indole formation:	–	
	6. Hydrogen sulfide formation:	+	
	7. Starch hydrolysis:	–	
	8. Citrate utilization		
	Koser medium:	±	
	9. Inorganic nitrogen source utilization		
	$NaNO_3$:	–	
	$(NH_4)_2SO_4$:	+	
	10. Pigment formation		
	King A medium:	–	
	King B medium:	–	
	11. Urease:	–	
	12. Oxydase:	+	
	13. Catalase:	– or ±	
	14. Growth conditions		
	pH: 4.1–7.5		
	Temp: 30–60		
	15. Attitude to oxygen: slightly aerobic		
	16. Oxidation–fermentation test: Fermentation		
	17. Acid and gas formation from sugar		
		Acid	Gas
	l-Arabinose:	–	–
	d-Xylose:	+	–
	d-Glucose:	+	–
	d-Mannose:	+	–
	d-Fructose:	+	–
	d-Galactose:	+	–
	Maltose:	+	–
	Sucrose:	+	–
	Lactose:	–	–
	Trehalose:	+	–
	d-Sorbitol:	–	–
	d-Mannitol:	+	–
	Inositol:	+	–
	Glycerol:	+	–
	Starch:	–	–
(D) Other properties			
	Mol% G+C of the DNA:	40.6%	

formed in the mixture was determined by gas chromatography described above.

One unit of nitrile hydratase was defined as the amount of bacterial cells or enzyme solution which catalyzed the formation of 1 μmol of propionamide per min. Specific activity was expressed as units per mg of dry cell weight.

Cultivation of strain SC-J05-1

Cells were grown aerobically at 45°C for 24 h with reciprocal shaking in the test tube containing 10 ml of a medium consisting of (per L): 10 g glycerol, 3.64 g polypeptone, 2.18 g yeast extract, 2.18 g malt extract, 6 g KH_2PO_4, 4 g K_2HPO_4, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg $ZnSO_4 \cdot 7H_2O$, 10 mg $MnSO_4 \cdot 7H_2O$, 10 mg $CoCl_2 \cdot 6H_2O$ (pH 6.5). The seed culture (5 ml) was inoculated into a 3-L jar fermentor containing 1.8 L of the same medium, and cultivated aerobically at 45°C for 24 h. The agitation speed and aeration rate were maintained at 500 rpm and 1.0 vvm, respectively. Growth was followed turbidimetrically by measuring absorbance at 660 nm.

Purification of nitrile hydratase

Preparation of cell-free extract: The cells of strain SC-J05-1, cultivated under the conditions described above, were harvested by centrifugation at 12 000 × *g* for 30 min. These cells were suspended in 100 mM potassium phosphate buffer (pH 7.0) and lysed using lysozyme (final concentration 0.5 mg ml^{-1}) for 1 h at 30°C. The lysate was centrifuged at 100 000 × *g* for 1 h, and the supernatant solution was collected.

Phenyl Sepharose HP chromatography: The cell-free extract was brought to 1.5 M ammonium sulfate, and applied to a Phenyl Sepharose HP column (bed volume: 196 ml) which had been equilibrated with 1.5 M ammonium sulfate on a FPLC system (Pharmacia, Uppsala, Sweden). Elution was carried out with a linear gradient of ammonium sulfate (1.5 to 0 M) in 50 mM potassium phosphate buffer (pH 7.0) at a flow rate of 15 ml min^{-1}. The active fractions were collected, concentrated by ultrafiltration, and dialyzed against 50 mM potassium phosphate buffer (pH 7.0).

Ion exchange chromatography: The enzyme solution was applied to a Q Sepharose Fast Flow column (bed volume: 294 ml) equilibrated with 20 mM bis tris propane/HCl buffer (pH 7.0). Elution was carried out with a linear gradient of 0–1.0 M NaCl in 20 mM bis tris propane/HCl buffer (pH 7.0). The active fractions were collected, concentrated by ultrafiltration, and dialyzed against 50 mM potassium phosphate buffer (pH 7.0).

Determination of molecular weight

The molecular weight of the enzyme was determined by gel filtration with a Superdex 200 prep grade column. Standard marker proteins used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa). The subunit molecular weight of the enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with the following marker proteins: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Substrate specificity

An aqueous solution of each nitrile was mixed with the purified nitrile hydratase, and incubated at 10°C for 5–60 min. Nitrile at 3, 5 or 50 mM was used in the reaction mixture. The reaction rates were determined by quantifying the amide produced or the nitrile consumed with GC and HPLC.

Effect of various compounds on the activity of nitrile hydratase

The purified enzyme solution was treated with each compound for 20 min at 20°C. The concentration of compounds was 1 mM in the reaction mixture except for $HgCl_2$ (0.01 mM). Residual activities were measured after treatment following the assay method described above.

Metal analysis

Metal ion content of the purified nitrile hydratase from strain SC-J05-1 was determined using an inductively coupled radiofrequency plasma spectrophotometer (SPS-1200VR, Seiko Instruments, Chiba, Japan). The sample used for analysis contained 1 mg ml^{-1} of the purified nitrile hydratase of strain SC-J05-1.

Chemicals and microorganisms

Phenyl Sepharose HP, Q Sepharose and Superdex 200 prep grade for purification were obtained from Pharmacia (Uppsala, Sweden). Standard proteins were also obtained from Pharmacia. Polypeptone was purchased from Nippon seiyaku (Osaka, Japan), yeast extract, malt extract and Bact Nutrient Broth were from Difco (Detroit, MI, USA). Nitriles, amides, and bis tris propane were from Nacalai tesque (Kyoto, Japan). The type strain of *Bacillus smithii* was purchased from LMG (Laboratorium Microbiologie Rijksuniversiteit Gent, Belgium).

Results

Screening and identification of strain SC-J05-1

Screening for the thermophiles having nitrile hydratase was performed on our collection of thermophiles (about 130 strains) that had been isolated from 100 soil or water samples collected near hot springs or volcanic areas exposed to high temperature and selected for growth at 55°C in the nutrient medium. The acrylamide-forming ability of each strain was examined using the intact cells with acrylonitrile as a substrate. Strain SC-J05-1 was found to have amide-forming ability.

The characteristics of strain SC-J05-1 are summarized in Table 1. The cells were aerobic rods, motile, spore-forming, and significant growth occurred at temperatures between 30–60°C. Though the strain SC-J05-1 was identified with

Table 2 Purification of nitrile hydratase from *Bacillus smithii* SC-J05-1

Purification steps		Cell-free extract	Phenyl Sepharose HP	Q Sepharose FF
Total protein	(mg)	1375	216	77.4
Total activity	(U)	6470	3700	2460
Specific activity	(U mg^{-1})	4.7	17.2	32.1
Purification	(-fold)	(1)	3.6	6.8
Activity yield	(%)	(100)	57	38

Purification means the relative value to the total specific activity of cell-free extract.
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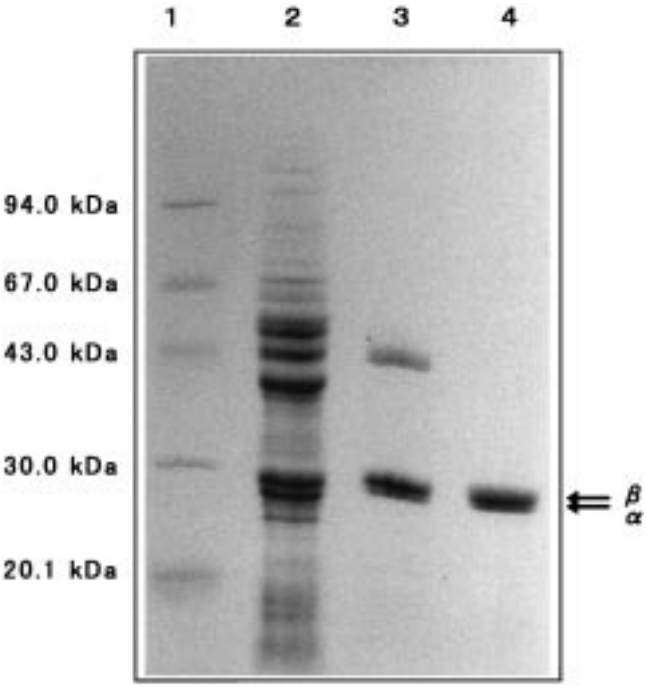

Figure 2 SDS-PAGE of purification steps of nitrile hydratase from *B. smithii* SC-J05-1. Lane 1, standard proteins, phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa); 2, cell-free extract; 3, Phenyl Sepharose HP; 4, Q Sepharose FF.

the genus *Bacillus* by consulting with Bergey's Manual of Systematic Bacteriology and Bergey's Manual of Determinative Bacteriology [7,16], no convincing correspondence in its characteristics with any described species was found. Recent reports on the taxonomy of *Bacilli* show the characteristics of strain SC-J05-1 were consistent with those of the newly established *B. smithii* [14]. A homology value of 95% was obtained by the total DNA–DNA hybridization test [5] between SC-J05-1 and the type strain of *B. smithii* (LMG12526) and identified SC-J05-1 as a strain of *B. smithii*.

Purification of nitrile hydratase

Purification of nitrile hydratase from the microbial cells of *B. smithii* SC-J05-1 is summarized in Table 2. The enzyme was purified 6.8-fold with a yield of 38% from the cell-free extract. The specific activity of the purified enzyme was 32.1 U mg^{-1} protein.

Molecular mass and subunit structure

The molecular mass of the purified native nitrile hydratase was estimated to be about 130 kDa from its mobility relative to the standard proteins on gel filtration by HPLC. The results of SDS-PAGE analysis of the samples from each step of the purification are shown in Figure 2. Two closely adjacent protein bands were recognized on SDS-PAGE of the final step sample, suggesting that the enzyme has two kinds of subunits differing in size. The molecular mass of these subunits of the nitrile hydratase were estimated to be 25.6 kDa (α subunit) and 26.8 kDa (β subunit) from the relative mobility to the standard proteins on SDS-PAGE. The relative amounts of the two types of subunit appeared equal. These results suggest that the nitrile hydratase of *B. smithii* SC-J05-1 consists of two α subunits and two β subunits.

Effect of temperature

The optimal temperature of the nitrile hydratase of SC-J05-1 was 40°C (Figure 3a) at pH 7.7. The inactivation of the enzyme was not detected up to the temperature of 50°C after incubation for 30 min at pH 7.0 (Figure 3b).

Effect of pH

The effect of pH on the activity of nitrile hydratase from SC-J05-1 was examined in the pH range of 5.0–10.5. Samples were incubated at 10°C for 10 min. The maximum

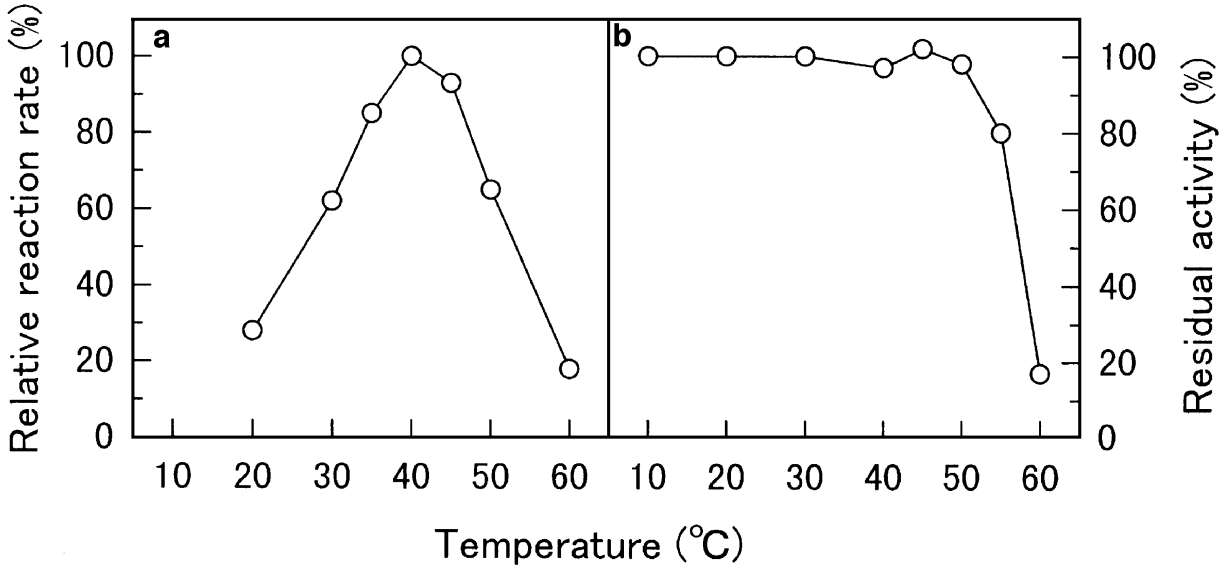

Figure 3 Effect of temperature on nitrile hydratase. (a) Initial reaction rate. The reaction was carried out with the purified nitrile hydratase of *B. smithii* SC-J05-1 for 10 min at pH 7.7 at various temperatures using propionitrile. (b) Thermostability. The purified nitrile hydratase was incubated for 30 min at the indicated temperatures. The residual activity was measured under the standard conditions at 10°C.

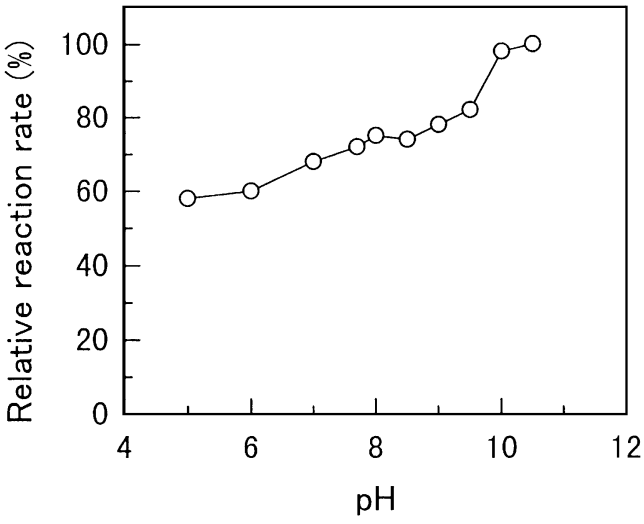

Figure 4 Effect of pH on initial reaction rate of nitrile hydratase. The reaction was carried out with the purified nitrile hydratase for 10 min at 10°C at various pHs using propionitrile.

value of the initial reaction rate was obtained at the highest pH (10.5 in these experiments) (Figure 4). The optimal pH of this enzyme appears to be in the alkaline region above pH 10.5.

The inactivation of the enzyme was not detected over the pH range of 6–9 after incubation for 23 h at 30°C (data not shown).

Substrate specificity

The substrate specificity was examined using several nitrile compounds with the purified nitrile hydratase (Table 3). The enzyme has a wide substrate specificity. Acetonitrile, acrylonitrile and butyronitrile were especially good substrates for this enzyme. Propionitrile, *n*-valeronitrile and 2-chloropropionitrile were relatively suitable substrates. Isobutyronitrile, crotononitrile, adiponitrile, succinonitrile, benzonitrile and 2-cyanopyridine were also hydrated by this enzyme.

Table 3 Substrate specificity of nitrile hydratase

Substrate	Relative activity (%)
Propionitrile	100
Acetonitrile	540
Acrylonitrile	390
n-Butyronitrile	290
n-Valeronitrile	240
2-Chloropropionitrile	38
Isobutyronitrile	23
Crotononitrile	23
Adiponitrile	7
Cyanopyridine	4
Succinonitrile	4
Benzonitrile	1

An aqueous solution of each nitrile was mixed with the purified nitrile hydratase, and incubated at 10°C for 5–60 min. Nitrile at 3, 5 or 50 mM was used in the reaction mixture. The reaction rate was determined by quantifying the amide produced or the nitrile consumed. Specific activity on propionitrile as substrate was 32.1 U mg^{-1} protein.

Inhibitors

The effects of various compounds were examined on the activity of nitrile hydratase using propionitrile as substrate (Table 4). The enzyme was sensitive to $AgNO_3$, $CuSO_4$, and $HgCl_2$ but insensitive to 5,5′-dithiobis(2-nitrobenzoic acid) and *N*-ethylmaleimide, which are thiol reagents. It was sensitive to hydroxylamine and phenylhydrazine, but insensitive to penicillamin and cycloserine, which are carbonyl reagents. Phenylmethylsulphonyl fluoride (which attacks serine residues), dithiothreitol and 2-mercaptoethanol (reducing reagents), and ammonium persulfate (oxidizing reagent) did not inhibit this enzyme activity. Chelating reagents such as EDTA, 1,10-phenanthroline and NaN_3 also had no effect on the enzyme activity.

Prosthetic group

Qualitative analysis of metal ions present in the enzyme was performed using the enzyme solution. The enzyme concentration in used solution was 1.0 mg ml^{-1} measured by the Bradford method or 0.57 mg ml^{-1} measured by absorbance at 280 nm. Cobalt ion was contained at a concentration of 0.65 μg ml^{-1} in this enzyme solution. The number of cobalt ions was calculated to be 1.5 or 2.5 mol mol^{-1} enzyme. No other transition metals were detected.

Discussion

Our goal was to find a new thermophilic microorganism having amide-forming ability by screening moderate thermophiles isolated from soil samples which had been collected near hot springs and volcanic areas. We found a novel thermophile *B. smithii* strain SC-J05-1 that produces nitrile hydratase. The temperature of 55°C was thought to be appropriate for the selection of moderate thermophiles.

B. smithii is a new species established by re-classification

Table 4 Effect of various compounds on the activity of nitrile hydratase

Compounds	Residual activity (%)
$CuSO_4$	40
$AgNO_3$	6.3
$HgCl_2$	46
5,5′-dithiobis(2-nitrobenzoic acid)	95
N-Ethylmaleimide	100
Iodoacetic acid	100
Hydroxylamine	83
Phenylhydrazine	8.2
l-Penicillamine	99
d-Penicillamine	95
d-Cycloserine	97
EDTA	101
1,10-Phenanthroline	103
NaN_3	99
Dithiothreitol	101
2-Mercaptoethanol	97
Ammonium persulfate	106

The purified enzyme solution was treated with each compound for 20 min at 20°C. The concentration of compounds was 1.0 mM in the reaction mixture except for $HgCl_2$, which was added at 0.01 mM. After treatment, residual activities were mesaured under the standard conditions using propionitrile at 10°C.

Table 5 Subunit composition and co-factor of nitrile hydratases

Strain[a]	Molecular weight (kDa)	Subunit size α(kDa)	β(kDa)	Subunit composition	Co-factor	Reference
SC-J05-1	130	26	29	$2\alpha2\beta$	Co	this work
J1 (H-NHase)	500–530	26	29	$10\alpha10\beta$	Co	[13]
J1 (L-NHase)	100	26	29	$2\alpha2\beta$	Co	unpublished
N-774	70.0	28.5	29.0	$2\alpha2\beta$	Fe	[4]
B23	100	25	25	$2\alpha2\beta$	Fe	[10]
C5	61.4	26.9		2α	Fe	[17]
R.sp	52	23	26	$\alpha\beta$	unknown	[6]
J-1	420	25	27	unknown	unknown	[1]

[a] SC-J05-1, *Bacillus smithii*; J1, *Rhodococcus rhodochrous*; N-774, *Rhodococcus* sp; B23, *Pseudomonas chlororaphis*; C5, *Corynebacterium* sp; R.sp, *Rhodococcus* sp; J-1, *Arthrobacter* sp.

of a thermophilic strain previously called *Bacillus coagulans* [14]. Twenty-six strains were moved to the new species. The distinctive characteristics of *B. smithii* are that it is a moderate thermophile which is able to grow at pH 5–7 and that the mol% G+C of the DNA is lower than that of other moderate thermophilic *Bacillus* strains.

B. smithii SC-J05-1 was able to assimilate various nitrile compounds as a sole nitrogen source (data not shown). The wide substrate specificity of the nitrile hydratase from *B. smithii* SC-J05-1 could account for this nitrile-assimilating ability of the microorganism. *B. smithii* SC-J05-1 was also able to assimilate the corresponding amides. So, it is thought to have the metabolic pathway of nitrile compounds by nitrile hydratase and amidase.

The nitrile hydratase was purified to homogeneity from the cells of *B. smithii* SC-J05-1. The nitrile hydratase of *B. smithii* SC-J05-1 was thermostable compared to other nitrile hydratases previously reported [1,6,10,13,17]. Thermostability would be expected to provide stability in storage. Some nitrile hydratases previously known need addition of *n*-butyrate [10,13] or isovalerate [17] to enzyme solutions to reduce spontaneous inactivation during enzyme purification. No inactivation of the nitrile hydratase of *B. smithii* SC-J05-1 was observed, though the purification process was performed at room temperature without stabilizers. Furthermore, purified nitrile hydratase did not inactivate during storage for 40 days at 4°C in 50 mM potassium phosphate buffer (pH 7.0).

Nitrile hydratases previously reported were metalloenzymes having Fe or Co ions (Table 5), and one metal ion was contained in each α subunit of those nitrile hydratases. The results of SDS-PAGE and gel filtration suggested that the nitrile hydratase of *B. smithii* SC-J05-1 was composed of $2\alpha2\beta$ subunits. Analysis of metal ions revealed that this enzyme contained two cobalt ions per molecule. So, *B. smithii* enzyme was thought to have a composition of $\alpha_2\beta_2Co_2$ similar to others.

Examination with various enzyme inhibitors could not demonstrate that the thiol group and carbonyl group of amino acid residues of the *B. smithii* SC-J05-1 enzyme were responsible for hydration of the nitrile because the enzyme was insensitive to some thiol and carbonyl reagents. But the presence of the thiol group in the catalytic center of *B. smithii* SC-J05-1 enzyme should not be also denied by these results. It was recently reported that ESR (electron spin resonance) spectra of the Fe-containing enzyme of *Pseudomonas chlororaphis* B23 [11] and EPR (electron paramagnetic resonance) spectra of the Co-containing enzyme of *Rhodococcus rhodochrous* J1 [3] suggested the presence of the thiol group in their catalytic centers, though these enzymes were also insensitive to 5,5′-dithiobis(2-nitrobenzoic acid), *N*-ethylmaleimide and sensitive to $AgNO_3$, $CuSO_4$, and $HgCl_2$ [10,13]. The fact that the *B. smithii* enzyme was insensitive to chelating reagents suggested that the cobalt ions were bound to the enzyme as tightly as *Rhodococcus rhodochrous* J1 enzyme [13].

The nitrile hydratase from *B. smithii* SC-J05-1 is of interest since its thermostability is superior to other nitrile hydratases from mesophiles and for some unique characters. It is expected that this enzyme will be advantageous in processes for the bioconversion of nitriles to amides.

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