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Nitrile hydratase of *Rhodococcus* sp. N-774

Purification and amino acid sequences

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The nitrile hydratase of *Rhodococcus* sp. N-774 was purified and crystallized. The enzyme is composed of two different subunits (molecular masses: subunit α, 28 500 Da; subunit β, 29 000 Da). The amino-terminal amino acid sequence of each subunit was determined. There is no sequence homology between the two subunits, suggesting that the peptides originate from different cistrons. The activity of the purified enzyme did not decrease during incubation in the dark, whereas it gradually decreased in intact cells.

Nitrile hydratase; Bacteria; Amino acid sequence

1. INTRODUCTION

NHase catalyzes the hydration of nitriles into the corresponding amides. In order to develop an enzymatic process for the production of acrylamide from acrylonitrile, we performed extensive screening of microorganisms exhibiting NHase activity and found *Rhodococcus* sp. N-774 [1]. An industrial process for the production of acrylamide has been established involving this strain [2]. Irradiation of producing cells or crude extracts with near-ultra violet light was found to cause marked enhancement of the enzyme activity [3]. Other NHases, from *Pseudomonas chlororaphis* B23 and *Brevibacterium* R312, were purified and characterized by Nagasawa et al. [4,5]. The molecular mass of the NHase isolated from *P. chlororaphis* B23 is 100000 Da, the enzyme being composed of four subunits with the same molecular size. On the other hand, the NHase of *Brevibacterium* R312 (molecular mass 85000 Da) is composed of two kinds of subunits. Both enzymes contain pyrroloquinoline quinone and non-heme iron [6]. This paper deals with the purification and amino acid sequences of the NHase from *Rhodococcus* sp. N-774.

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Abbreviations: NHase, nitrile hydratase; HPLC, high-performance liquid chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Chemicals*

All nitriles, amides and various acids, and 2-mercaptoethanol were of reagent grade and purchased from Tokyo Kasei Kogyo Co. Bovine serum albumin, trifluoroacetic acid and sodium dodecyl sulfate were obtained from Wako Pure Chemical Industries Ltd. Triethanolamine was purchased from Hikotaro Shudzui Co.

2.2. *Microorganism and cultural conditions*

Rhodococcus sp. N-774 was cultured on an industrial scale and a portion of the culture was used as the source of the NHase. The cultural conditions for *Rhodococcus* sp. N-774 were given in the previous paper [2].

2.3. *Enzyme assay*

The standard reaction mixture (1.5 ml) contained 200 μmol of *n*-propionitrile, 40 μmol of Na_2HPO_4/KH_2PO_4 buffer (pH 7.3) and an appropriate amount of enzyme. The reaction was initiated by addition of the enzyme and incubation was carried

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out at 20°C for 10 min. The reaction was stopped by adding 0.5 ml of 2 N HCl. *n*-Propionamide formed was determined by gas chromatography (Shimadzu, GC-6AM) packed with Porapak PS (Waters Associates Inc.). The analytical conditions were given previously [1]. When *n*-propionic acid was determined with the same system, the column temperature was changed to 145°C. One unit of propionamide-forming activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol propionamide per min. The protein concentration of the NHase solution was determined by the method of Lowry et al. [7].

2.4. *Enzyme purification*

2.4.1. Step 1, preparation of a cell free extract and ammonium sulfate fractionation

The enzyme was highly unstable, therefore, all procedures were carried out at 0 to 4°C in 0.05 M Na_2HPO_4/KH_2PO_4 buffer (pH 6.5) containing 40 mM sodium *n*-butyrate (buffer A), as reported by Nagasawa et al. [4,5]. Cells (72 g wet wt) were suspended in buffer A and then disrupted twice with a French Press operating at 1500 kg/cm^2. Cells and debris were removed by centrifugation at 10000 × *g* for 30 min. Proteins in the supernatant were precipitated with ammonium sulfate at between 30 and 60% saturation. The precipitate was solubilized in buffer A and ammonium sulfate was removed by dialysis against the same buffer.

2.4.2. Step 2, DEAE-Cellulofine column chromatography

The dialyzed solution was applied to a DEAE-Cellulofine (AM-type; Seikagaku Kogyo Co.) column, 5 × 25 cm, which was developed with an increasing linear gradient of NaCl, from 0 to 1 M, in buffer A. The active fractions eluted at 0.15 to 0.35 M NaCl were combined and then diluted twice with buffer A. The 2nd DEAE-Cellulofine chromatography was carried out by simply applying the enzyme solution on an activated column again, with elution with an increasing stepwise gradient of NaCl (0.15, 0.25 and 0.35 M) in buffer A. The active fractions eluting at 0.25 M NaCl were combined.

2.4.3. Step 3, phenyl-Sepharose chromatography

Ammonium sulfate was added to 25% saturation to the above active fractions, which were then applied to a phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals) column, 5 × 25 cm, equilibrated with buffer A 25% saturated with ammonium sulfate. The column was developed with a stepwise reduction in the ammonium sulfate concentration (15, 10, 5 and 0% saturation) and the NHase was eluted at 5% ammonium sulfate saturation.

2.4.4. Step 4, gel filtration on Sephadex G-150

The enzyme in the active fraction was precipitated at 60% ammonium sulfate saturation. The precipitate was dissolved in a small amount of buffer A and then passed through a Sephadex G-150 (Pharmacia Fine Chemicals) column, 1.5 × 100 cm, equilibrated with buffer A.

2.4.5. Step 5, octyl-Sepharose chromatography

A portion of the active fraction, eluted from Sephadex G-150, was adjusted to 25% ammonium sulfate saturation and then loaded on an octyl-Sepharose CL-4B (Pharmacia Fine Chemicals) column, 3 × 50 cm, equilibrated with buffer A 25% saturated with ammonium sulfate. The enzyme was eluted with buffer A 10% saturated with ammonium sulfate after extensive washing of the column with the buffer used for equilibration. The active fraction was dialyzed against buffer A for removal of the salt and then it was kept at 0°C at the protein concentration of 12.4 mg/ml. This fraction was used for all researches unless otherwise mentioned.

2.4.6. Step 6, crystallization of the enzyme

A portion of the active fraction (50 mg protein), obtained on octyl-Sepharose chromatography, was dialyzed against 0.1 M K_2HPO_4/KH_2PO_4 buffer (pH 7.0) containing 20 mM potassium *n*-butyrate. The protein concentration was adjusted to 22.5 mg/ml and crystallization was performed by the addition of crystalline ammonium sulfate at 0°C.

2.5. *Analytical electrophoresis*

SDS-PAGE was performed in a 1 mm thick slab gel, containing 18% acrylamide and 0.48% *N,N'*-methylenebisacrylamide, by the method of Laemmli [8].

2.6. *Separation of subunits*

The separation of subunits of NHase was carried out with an HPLC apparatus using TRIROTAR (Japan Spectroscopic Co.). The NHase was separated into two subunits at room temperature on a C_4 reverse-phase column (Senshu Pak VP-304-1251, 0.46 × 25 cm; Senshu Scientific Co.), with an increasing linear gradient of acetonitrile, between 20 and 70%, at the flow rate of 1.5 ml/min. Trifluoroacetic acid was always present at the concentration of 0.1% in the running solution. The subunit fractions were freeze-dried and then used for further studies.

2.7. *Molecular mass determination by HPLC*

The molecular masses of the native enzyme and its subunits were determined by HPLC on a molecular sieve column (TSK-gel G3000 SW XL; Toyo Soda Manufacturing Co. Ltd). The eluent used was 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0) containing 0.2 M KCl and 10 mM potassium *n*-butyrate. The flow rate and the column temperature were 25°C and 0.7 ml/min, respectively. The marker proteins used comprised M.W.-Marker (Oriental Yeast Co.), which is a mixture of the following proteins: glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome *c* (12.4 kDa).

2.8. *Amino acid sequence*

A gas-phase amino acid sequencer (Applied Biosystems, model 470A) was used for amino-terminal amino acid analysis and sequencing.

3. RESULTS AND DISCUSSION

3.1. *Enzyme purification*

The NHase was purified about 11.5-fold, with a yield of 11%, from the cell-free extract (table 1), and a photomicrograph of the crystallized enzyme is presented in fig.1. The crystallized enzyme

Table 1

Purification of NHase isolated from *Rhodococcus* sp. N-774

	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude extract	17165	110	100	1
Fractionation with $(NH_4)_2SO_4$	7872	180	75	1.6
DEAE-Cellulofine, twice	1470	690	54	6.3
Phenyl-Sepharose	758	1110	45	10.1
Sephadex G-150	657	1220	42	11.1
Octyl-Sepharose	258	1270	17	11.5
Crystallization	160	1260	11	11.5

catalyzed the hydration of propionitrile, at 1270 U/mg protein, under the standard reaction conditions. The specific activity of the final preparation did not change on further crystallization. On polyacrylamide gel electrophoresis, the purified enzyme migrated as a single species, as judged on staining for protein of the polyacrylamide gel.

3.2. *Subunit composition*

When the enzyme preparation obtained on octyl-Sepharose chromatography was analyzed by SDS-PAGE, two closely adjacent protein bands were observed, suggesting the presence of two subunits of different sizes (fig.2). These two

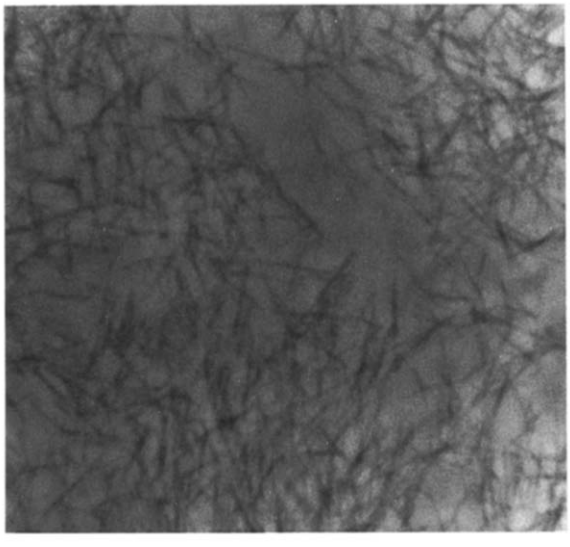

Fig.1. Photomicrograph of crystalline NHase (× 1500).

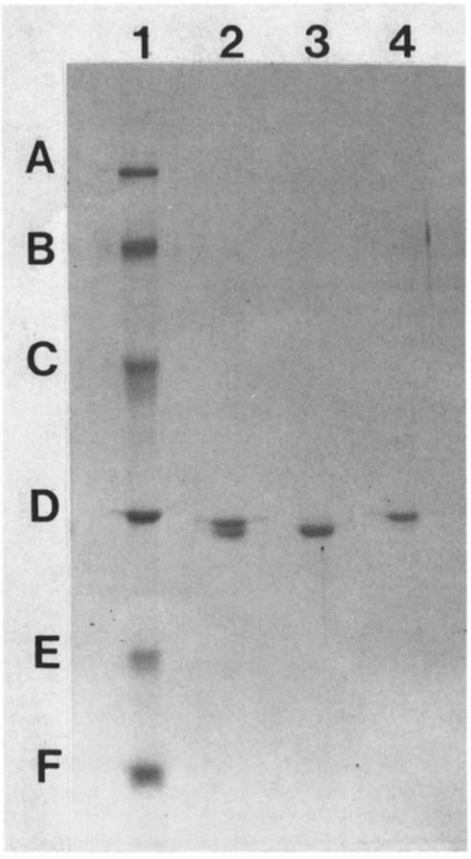

Fig.2. SDS-PAGE of purified NHase and its subunits. Octyl-Sepharose-purified NHase (lane 2, 3 μg protein) and its subunits (lane 3: subunit α, 1.5 μg protein, and lane 4: subunit β, 1.5 μg protein) were compared as to their molecular masses. The marker proteins used were as follows: (A) phosphorylase *b*, 94 kDa; (B) albumin, 67 kDa; (C) ovalbumin, 43 kDa; (D) carbonic anhydrase, 30 kDa; (E) trypsin inhibitor, 20.1 kDa; (F) α-lactalbumin, 14.4 kDa.

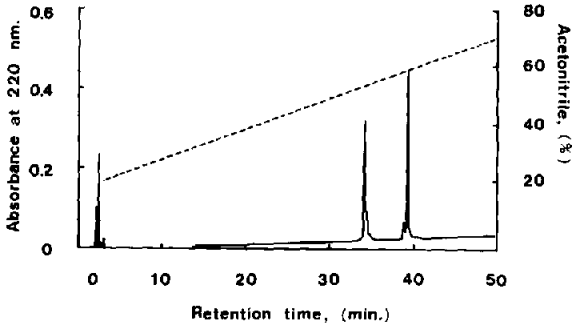

Fig.3. Separation of NHase into subunits by HPLC. 50 μg of NHase was loaded on a C_4 reverse-phase column (0.46 × 25 cm). Absorbance at 220 nm (——); acetonitrile concentration (– – –).

Table 2

Amino-terminal amino acid sequences of subunits α and β

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Subunit α	H_2N-Ser-	Val-	Thr-	Ile-	Asp-	His-	Thr-	Thr-	Glu-	Asn-	Ala-	Ala-	Pro-	Ala-	Gln-	Ala-	Pro-	Val-	Thr
Subunit β	H_2N-Met-	Asp-	Gly-	Val-	His-	Asp-	Leu-	Ala-	Gly-	Val-	Gln-	Gly-	Phe-	Gly-	Lys-	Val-	Pro-	His-	Thr

subunits were separated by HPLC on a C_4 reverse-phase column (fig.3) and then the molecular mass of each one was determined by SDS-PAGE (fig.2). The smaller and bigger subunits were named subunit α (molecular mass, 28500 Da) and subunit β (molecular mass, 29000 Da), respectively. The molecular mass of the holoenzyme of NHase was determined to be 70000 Da by gel filtration HPLC.

3.3. *Amino-terminal amino acid sequences of the subunits*

The amino-terminal amino acids of the subunits of the NHase were analyzed with a gas-phase amino acid sequencer. As shown in table 2, there is no sequence homology between the two subunits, suggesting that the peptides originate from different cistrons.

3.4. *Effect of light on the enzyme activity*

The NHase activity of intact cells of *Rhodococcus* sp. N-774 increases significantly on light irradiation and gradually decreases in the dark, but the activity of *P. chlororaphis* B23 does not change with light, as reported previously [3]. In this work, we examined as to whether or not the activity of the purified enzyme is also lost in the dark, as follows. The NHase was kept in the dark for a week at 0°C in buffer A, at the protein concentration of 124 μg/ml. A portion of the enzyme was taken out and then left under room light for 1 h at 0°C just before the enzyme assay. The other portion was kept continuously in the dark and the assay was performed in the dark using the standard reaction mixture. No prominent difference was observed in activity between the enzyme kept in continuous dark (950 U/mg) and the light-treated enzyme (990 U/mg).

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