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ORIGINAL ARTICLE

Purification of a hyperactive nitrile hydratase from resting cells of *Rhodococcus rhodochrous* PA-34

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Abstract Apropionitrile-inducednitrilehydratase(NHase), a promising biocatalyst for synthesis of organic amides has been purified from cell-free extract of *Rhodococcus rhodochrous* PA-34. About 11-fold purification of NHase was achieved with 52% yield. The SDS-PAGE of the purified enzyme revealed that it consisted of two subunits of 25.04 kD and 30.6 kD. However, the molecular weight of holoenzyme was speculated to be 86 kD by native-PAGE. This NHase exhibited maximum activity at pH 8.0 and temperature 40°C. Half-life was 2 h at 40°C and 0.5 h at 50°C. The Km and Vmax were 167 mM and 250 μmole/min/mg using 25 mM 3-cyanopyridine as substrate. $AgNO_3$, $Pb(CH_3COO)_2$ and $HgCl_2$ inhibited the NHase to extent of 89–100%.

Keywords *Rhodococcus rhodochrous* PA-34 · Nitrile hydratase (NHase) · Purification · 3-Cyanopyridine · Nicotinamide

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Introduction

Nitriles are cyano group-containing organic compounds and important starting materials for synthesis of a range of commercially important amides and acids. The chemical processes of nitrile transformation have certain limitations such as multistep reaction, production of toxic by-products, low level of substrate selectivity (chemo-, regio- and stereo-), often require harsh conditions (very high or low pH) and high-energy inputs, which lead to increase the cost of synthesis. However, use of enzymes in organic synthesis has overcome many of such limitation of chemical processes. Nitrilase, nitrile hydratase and amidase are three important enzymes which catalyse the hydrolysis of nitriles to corresponding amides or acids. Out of these three enzymes, nitrile hydratase (NHase, EC 4.2.1.84) catalyses the hydration of nitriles to related amides, was first reported in 1980 by Asano et al. [1]. Nitrile hydratase has emerged as enzyme of choice in chemical industries practicing white biotechnology for the organic synthesis. Currently, acrylamide, nicotinamide and 3-cyanovaleramide are being produced at industrial scale using the immobilized resting cells of NHase producing organism [2]. We have earlier reported a hyperactive propionitrile-induced NHase of *R. rhodochrous* PA-34 which have been used to synthesise acrylamide (600 g/l), butyramide (585 g/l) and nicotinamide (855 g/l) [3–5]. In the present communication, the purification and characterization of this NHase of *R. rhodochrous* PA-34 are being reported.

Materials and methods

Materials: Pre-packed gel filtration column (HiPrep™ Sephacryl S-300 HR) and ion-exchange column (HiPrep™

DEAE-Sepharose FF), native-polyacrylamide gel electrophoresis (PAGE) protein molecular size markers and reagents of electrophoresis were purchased from Amersham Pharmacia Biotech, Sweden. Sodium dodecyl sulphate (SDS)-PAGE protein molecular size markers were procured from Genei Bangalore Ltd, Bangalore, India. Media ingredients were from Hi-Media, Mumbai, India and all other chemicals and reagents were of analytical grade. Purification of NHase of *R. rhodochrous* PA-34.

All the steps for purification of NHase were carried out at 4°C using 0.1 M potassium phosphate buffer containing 0.15 M NaCl (pH 7.5) and centrifugation was carried out at 25,000 g for 30 min at 4°C (if not otherwise stated). The enzyme preparations at various stages of NHase purification were subjected to SDS- and native-polyacrylamide gel electrophoresis (PAGE) and analyzed for protein concentration and enzyme activity. The enzyme was purified using following steps:

Preparation of cell free extract of R. rhodochrous PA-34: The *R. rhodochrous* PA-34 cells grown in 2L culture medium were harvested by centrifugation at 5000 g for 10 min and washed twice with 0.1 M potassium phosphate buffer pH 7.5 and suspended in 20 ml of same buffer [6]. Lysozyme (100 µl of 100 mg/ml) was added in cell suspension and stored at 4°C. After 12 h incubation, cell suspension was washed twice as above and suspended in 20 ml of buffer. It was homogenized by 60 cycles of ultrasonication (each cycle comprises 30 sec of sonication followed by 30 sec cooling) at 250 W and 19 Hz at 4°C and the homogenate was centrifuged. The NHase activity was assayed in supernatant (i.e. cell free extract, CFE) and pellet (i.e. the aggregate of cell debris and intact cells).

Ammonium sulphate fractionation: The CFE was subjected to various saturations of ammonium sulphate (10–80% saturation) and the precipitates were collected by centrifugation and suspended in buffer. The ammonium sulphate fraction (ASF) containing maximum NHase activity was processed further.

First Sephacryl S 300 HR column chromatography: The ASF was filtered through 0.45 µ filter and directly loaded on to pre-packed gel filtration column (HiPrep™ 16/60 Sephacryl S-300 High Resolution) equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 M NaCl. The gel filtration chromatography was performed using AKTA prime™ V2.00 at a flow rate of 0.3 ml/min of elution buffer (same as equilibration buffer). The fractions exhibiting NHase activity were pooled and precipitated by subjecting to 70% saturation of ammonium sulfate, and the precipitates were collected by centrifugation and suspended in the buffer (the enzyme preparation at this stage was designated as SCC1).

Second Sephacryl S 300 HR column chromatography: The enzyme suspension (SCC1) was then again applied on to a Sephacryl S-300 HR column keeping all conditions the same as before (the enzyme rich pooled fractions designated as SCC2).

DEAE FF column chromatography: The enzyme preparation (SCC2) was filtered through 0.45 µ filter and applied on to an ion-exchange chromatography column (HiPrep 16/10 DEAE FF) equilibrated with 0.1 M potassium phosphate buffer pH 7.5 containing 0.1 M NaCl. After loading the protein sample, column was washed with buffer to remove the unbound protein and then eluted with a linear gradient of NaCl from 0.1 to 0.7 M in 0.1 M potassium phosphate buffer. The flow rate was 1.0 ml/min and fractions of 3.0 ml were collected. The fractions exhibiting single band on native-PAGE were pooled and termed as purified NHase.

Characterization of purified NHase of *R. rhodochrous* PA-34

Effect of pH dependent stability of NHase: To study the effect of pH (4 to 10) on activity of purified NHase, it was incubated for 60 min at 25°C in following buffers (0.1 M): citric acid-sodium citrate (pH 4, 4.5, 5 and 5.5), potassium phosphate (pH 6, 6.5 and 7), Tris-HCl (pH 7.5, 8 and 8.5) and glycine-NaOH (pH 9, 9.5 and 10) and the residual activity was measured.

Effect of temperature on activity and stability of NHase: The effect of temperature (10°C to 70°C) on the activity was investigated in 0.1 M Tris-HCl buffer, pH 8 to find out the optimum temperature. The thermal stability of this enzyme was also assessed by treating purified NHase in 0.1 M potassium phosphate buffer, pH 8 at 40°C and 50°C for 0 to 6 h. During thermal exposure, the NHase activity was assayed at an interval of 0.5 h till 6 h.

Effect of metal ions and inhibitors: To assess the effect of metal ions and inhibitors (as mentioned in Result and discussion) on the activity of purified NHase, the enzyme was incubated with 1mM concentration of various compounds for 10 min at 25°C. The residual activity was assayed at optimum pH and temperature.

Determination of Km and Vmax: The Km and Vmax of NHase of *R. rhodochrous* PA-34 were graphically determined from the Lineweaver-Burk plot (1/v versus 1/[S]) using 3-cyanopyridine as substrate.

Nitrile hydratase assay: The activity of nitrile hydratase (NHase) was assayed in 1 ml reaction mixture containing 12.5 µg enzyme (crude or partially purified or purified), 0.025 M 3-cyanopyridine and 0.1 M potassium phosphate buffer, pH 8. It was incubated at 30°C for 30 min in a water bath shaker and the reaction was terminated by the addi-

tion of 1 ml of 0.1 M HCl. The concentration of substrate and product in the reaction mixture were analyzed by high performance liquid chromatography (HPLC) following the procedure reported earlier [6]. One unit of NHase activity (U) was defined as the amount of enzyme which catalyses the conversion of one μmole of 3-cyanopyridine to nicotinamide per min under the assay conditions.

Determination of protein concentration and polyacrylamide gel electrophoresis (PAGE): The protein concentration in the samples was measured according to Bradford's method [7]. The sodium dodecyl sulphate (SDS) and native-polyacrylamide gel electrophoresis (PAGE) were carried out according to Laemmli [8].

Results and discussion

The two liters culture of *R. rhodochrous* PA-34 yielded a cell mass of 4.96 g (on dry weight basis) with 11,600 U of NHase activity (i.e. 2.34 U/mg dry cell weight) against 3-cyanopyridine as substrate[6].

Preparation of cell free extract: In the present investigation, we could not break resting cell by ultrasonication. This might be due to peculiar chemical composition and rigid structure of cell walls of actinomyces (as the *R. rhodochrous* PA-34 belongs to this group of bacteria). Most of the bacterial cell walls are prone to hydrolytic action of lysozyme, but for hydrolysis of cell walls of *Rhodococcus* spp lysozyme and *Achromobacter* endopeptidase have been used [9–10]. The exposure of resting cells of *R. Rhodochrous* PA-34 to 0.5 mg lysozyme per ml cell suspension for 12 h followed by ultrasonication leads to disruption of cells. It seems that lysozyme had weakened the cell walls that facilitated lysis of *R. rhodochrous* PA-34 cells with ultrasonication. Even this resulted in release of 8.7% of the NHase activity (998 U) from the resting cells (containing 11,600 U). However, >90% activity was detected in aggregates of intact cell or cell debris after sonication. In spite of enzymatic treatment followed by ultrasonication, only a small percentage of *R. rhodochrous* PA-34 cells could be lysed. For purification and characterization of NHase even 8.7% enzyme activity recovered from the resting cells was sufficient. The lysozyme has been used for the lysis of cells for extraction of nucleic acids [9] but its use in conjunction with ultrasonication for lysis of *Rhodococcus* cells for the preparation of cell free extract for purification of intracellular enzyme is being reported for the first time. Further standardization of this method may improve the release of enzymes from the cells.

Purification of nitrile hydratase: The NHase was purified from the cell free extract of *R. rhodochrous* PA-34 using four steps of purification as summarized in Table 1. The cell free extract (CFE) had a specific activity of 4.16 U/mg protein and its subjection to 0 to 70% saturation of ammonium sulphate precipitated maximum NHase activity (5.24 U/mg protein) and was termed as ASF with an yield of 84%. The gel filtration of ASF using Sephacryl S-300 HR and pooling together of NHase rich fractions increased specific activity of the NHase preparation (SCC1) to 7.99 U/mg protein and the enzyme yield was 77%. The enzyme preparation after second Sephcryl S-300 HR gel chromatography (SCC2) of the SCC1 proved fruitful in further increasing the specific activity to 12.66 U/mg protein but the yield of the enzyme declined to 58% of the CFE.

The ion exchange chromatography of NHase rich fractions of SSC2 eluted with 0.42 to 0.52 M NaCl yielded a purified NHase (DEAE) of *R. rhodochrous* PA-34 with a specific activity of 46.83 U/mg protein and 52% yield. The elution of the NHase with 0.42 to 0.52 M NaCl in DEAE ion exchange chromatography revealed that the enzyme has a strong negative charge on it at pH 8. The NHase of *R. rhodochrous* PA-34 was purified up to 11.3-fold with an yield of 52% from the cell-free extract (Table 1) which was significantly high as compared to yield of purified NHases reported earlier except 56% purified NHase yield of *Bacillus pallidus* Dac 521 [11].

The purified NHase of *R. rhodochrous* PA-34 exhibited two bands of molecular mass of 30.6 and 25.04 kD on SDS-PAGE (Fig. 1a). It meant that this enzyme is an association of two different polypeptides. This feature of the NHase of

Table 1 Purification of nitrile hydratase of *R. rhodochrous* PA-34

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
CFE	998	240	4.16	100	-
ASF	838	160	5.24	84	1.3
SCC1	772	97	7.96	77	1.9
SCC2	582	46	12.66	58	3.0
DEAE	515	11	46.83	52	11.3

CFE: cell-free extract; ASF: ammonium sulphate fractionation; SCC1: first Sephacryl S-300 HR column chromatography; SCC2: second Sephacryl S-300 HR column chromatography and DEAE: FF column chromatography

R. rhodochrous PA-34 is similar to the previously reported NHases. These two polypeptides (subunits) of NHase were named as α-subunit and β-subunit. Usually, α-subunit is lighter in weight in comparison to β-subunit [12]. It means that the protein bands on SDS-PAGE of 25.04 kD and 30.6 kD are α- and β-subunit of NHase of *R. rhodochrous* PA-34, respectively. This enzyme showed a single band on native-PAGE of molecular mass of 86 kD (Fig. 1b). Therefore, we assumed that the functional unit of this enzyme was constituted by one α-subunit and two β-subunits (i.e. $\alpha\beta_2$). However, the number of the subunits of polypeptides (α- and β-subunit) varied from 2-20 in NHases of other micro-organism and the molecular mass of earlier reported active NHases varied from 54 kD to 505 kD [12, 13].

Characterization of purified NHase of *R. rhodochrous* PA-34

Effect of pH dependent stability of NHase: The optimum pH stability of purified NHase of *R. rhodochrous* PA-34 was 8, however, it was active within pH 5 to 10. Although, the optimum pH of previously reported NHases varied from 6 to 9 and most of them have pH optima around pH 7 [14]. The pH below 5 and above 10 drastically decreased the activity of purified NHase and it may be due to structural change of enzyme since a change in pH affect the ionization state of side chain of amino acid that may be involved in the maintenance of proper folding of protein needed for its catalytic activity. However, the resting cells of *R. rhodochrous* PA-34 showed the optimum NHase activity at wide pH range from 6.5 to 9.5 [6]. It showed that this enzyme was more stable within the cells.

Effect of temperature on activity and stability of NHase: The optimum temperature of *R. rhodochrous* PA-34 NHase was observed at 40°C. Below and above 40°C, NHase activity of *R. rhodochrous* PA-34 decreased drastically. Generally, the mesophilic NHases have exhibited maximum activity near ambient temperature between 20 and 40°C [14, 15]. However, thermophilic NHases of *Bacillus* RAPc8 [16] and *Pseudonocardia thermophila* [17] exhibited maximum activity at 60°C. The half-life of NHase of *R. rhodochrous* PA-34 was 2 h at 40°C, 0.5 h at 50°C and it was rapidly inactivated at temperature above 50°C. However, NHase of *B. pallidus* Dac 521 had half-life 0.85 h at 50°C [11]. The NHase within the resting cells of *R. rhodochrous* PA-34 showed wide optimum temperature from 30°C to 60°C, which further indicate that this enzyme was more stable within the cells.

Km and Vmax: The Km and Vmax values were 167 mM and 250 μmole/min/mg protein of purified NHase of *R. rhodochrous* PA-34 using 3-cyanopyridine as substrate. However, the Km and Vmax of L-NHase and H-NHase of *R. rhodochrous* J1 were 0.30 mM and 579 μmole/min/mg protein, and 200mM and 370 μmole/min/mg protein,

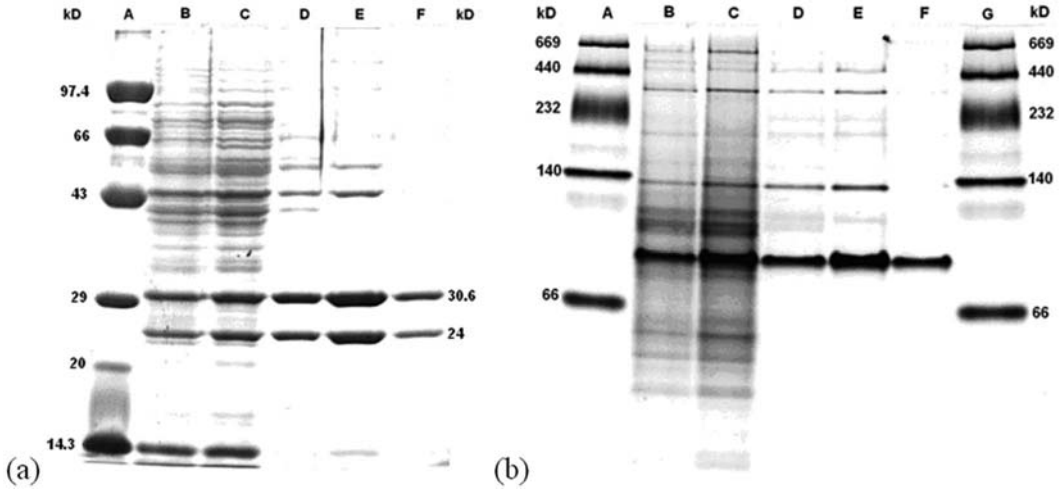

Fig. 1 (a) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of purified nitrile hydratase of *R. rhodochrous* PA-34. Lane A and G: protein molecular weight marker contained phosphorylase b (97.4 kD), bovine serum albumin (66.0 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), soybean trypsin inhibitor (20 kD) and lysozyme (14.3 kD). (b) Native-polyacrylamide gel electrophoresis of nitrile hydratase preparations at various stages of purification. Lane A and G were loaded with native-high molecular weight protein marker: thyroglobuline (669 kD), ferritin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD) and albumin (66 kD). Approximately 10 μg protein of cell free extract (lane B), 15 μg protein of the ammonium sulphate fraction (lane C), 8 μg protein of first Sephacryl S-300 HR column chromatography fraction (lane D), 10 μg protein of second Sephacryl S-300 HR column chromatography (lane E) and 4 μg protein of DEAE FF column chromatography fraction (lane F) were applied to the gel.

respectively [15].

Effect of metal ions and inhibitors: The effect of various metal ions and inhibitors on the NHase activity of *R. rhodochrous* PA-34 was studied (Table 2). Among ten metal ions tested, Ag^{+} ($Ag\ NO_3$) caused complete inhibition, while $Pb2^{+}$ ($Pb(CH_3COO)_2$) and Hg^{2+} ($HgCl_2$) inhibited the NHase to extent of 95 and 89% respectively. This meant that cysteine is involved at the active site of the enzyme and these results supported the earlier findings [14].

Generally, the metalloproteins can be readily distinguished from the other classes of protein by treatment with metal chelating agents such as ethylene diamine tetra acetic acid (EDTA), which knock out metal ion cofactor and thus impairing the catalytic activity of enzyme. However, in the present study, EDTA could not so significantly reduce the activity of purified NHase of *R. rhodochrous* PA-34. It seems that Co^{2+} is either strongly bound to or is deeply embedded into the NHase protein.

Dithiothreitol and 2-mercaptoethanol are reducing agent and break the disulphide bonds between two cysteine residues affecting 3D structure of enzyme. However, we could not get the inactivation of NHase activity after treatment of DTT and 2-mercaptoethanol. Similarly, PMSF did not inhibit the enzyme activity. Ammonium persulphate slightly decreased the enzyme activity (about 5%) while urea which generally inhibits the enzyme activity by increasing the solubility of non-polar amino acid side-chain, did not affect the NHase activity of *R. rhodochrous* PA-34. In the presence of hydroxylamine, phenyl hydrazine, iodoacetic acid and L-ascorbic acid, the activity of this NHase declined to the extent of 55–70%.

Table 2 Effect of metal ions and compounds on purified nitrile hydratase of *R. rhodochrous* PA-34

Metal ions/ inhibitors (1 mM)	Residual relative activity (%)
None	100
$AgNO_3$	0
$CaCl_2$	90
$CdCl_2$	92
$CoCl_2$	98
$CuCl_2$	93
$FeCl_2$	95
$HgCl_2$	10.7
$MgCl_2$	96
$MnCl_2$	92
$(CH_3COO)_2\ Pd.3H_2O$	5.5
Ammonium persulphate	95.5
Dithiothreitol	100.9
EDTA	87.9
Hydroxylamine	37.4
Iodoacetic acid	35
L-Ascorbic acid	45.4
2-mercaptoethanol	110.5
Phenyl hydrazine	32
PMSF	108.7
Sodium azide	73.4
Urea	86.1

Purified NHase (12.5 μg) of *R. rhodochrous* PA-34 was first incubated in one ml of 1 mM metal salts or inhibitors at 25°C for 30 min and then its activity was assayed at 40°C in 0.1 M Tris-HCl buffer, pH 8.

Conclusion

A hyperactive nitrile hydratase of *R. rhodochrous* PA-34 of 86 kD has been purified from the cell free extract in four steps involving ammonium sulphate fractionation, Sephacryl S-300 column chromatography 1 and 2, and DEAE ion exchange chromatography. The treatment of resting cells of *R. rhodochrous* PA-34 with lysozyme made cells susceptible to disruption with ultrasonication for preparation of cells free extract. This resulted in 11-fold purification with a 52% yield of NHase from *R. rhodochrous* cells.

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