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Biochemical characterization of *Rhodococcus erythropolis* N′4 nitrile hydratase acting on 4-chloro-3-hydroxybutyronitrile

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ABSTRACT

The *Rhodococcus erythropolis* strain (N′4) possesses the ability to convert 4-chloro-3-hydroxybutyronitrile into the corresponding acid. This conversion was determined to be performed by its nitrile hydratase and amidase. Ammonium sulfate fractionation, DEAE ion exchange chromatography, and phenyl chromatography were used to partially purify nitrile hydratase from cell-free extract. A SDS-PAGE showed that the partially purified enzyme had two subunits and gel filtration chromatography showed that it consisted of four subunits of $\alpha_2\beta_2$. The purified enzyme had a high specific activity of 860 U mg^{-1} toward methacrylonitrile. The enzyme was found to have high activity at low temperature range, with a maximum activity occurring at 25 °C and be stable in the presence of organic acids at higher temperatures. The enzyme exhibited a preference for aliphatic saturated nitrile substrates over aliphatic unsaturated or aromatic ones. It was inhibited by sulfhydryl, oxidizing, and serine protease inhibitors, thus indicating that essential cysteine and serine residues can be found in the active site.

The purified nitrile hydratase was able to convert 4-chloro-3-hydroxybutyronitrile into the corresponding amide at 15 °C. GC analysis showed that the initial conversion rate of the reaction was 215 mg substrate consumed min^{-1} mg^{-1}. This demonstrated that this enzyme could be used in conjunction with a stereoselective amidase to synthesize ethyl (*S*)-4-chloro-3-hydroxybutyrate, an intermediate for a hypercholesterolemia drug, Atorvastatin.

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1. Introduction

While various nitrile compounds can be found in nature, many others have also been produced by the chemical and pharmaceutical industries as intermediates in organic synthesis [1,2]. Two different enzymatic pathways can be utilized to convert these nitrile compounds into the corresponding amides and acids. Nitrilase (EC 3.5.5.1) is an enzyme which catalyzes the direct hydrolysis of nitriles to the corresponding acids. Conversely, nitrile hydratase (NHase, EC 4.2.1.84) catalyzes the hydration of the nitriles into the corresponding amides, which is then followed by the amidase (EC 3.5.1.4) hydrolysis of the amides into the corresponding acids [3–7].

To this end, NHases, which have proven to be very practical industrial enzymes, have been employed in the large-scale production of acrylamide and nicotinamide [5,8–11]. In addition, many NHases taken from various microorganisms have been purified and characterized. These include *Rhodococcus* [2,9,12–14], *Arthrobacter* [15], *Pseudomonas* [8], *Bacillus* [5], and *Pseudonocardia* [16]. NHases can, in accordance with the composition of the metal ions, be classified into two groups, namely the Fe-type featuring a non-heme iron atom [2,8,10,17] and the Co-type which boasts a non-corrinoid cobalt atom [9,13,18,19]. Experiments have shown that while the Fe-type NHase prefers small aliphatic nitriles, the Co-type NHase is able to rapidly hydrolyze aromatic nitriles [2,8,9,13].

Lipitor (Atorvastatin calcium) is a synthetic lipid-lowering agent. Atorvastatin is an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis [20]. Previously nitrilase enzyme was used to catalyze desymmetrization of 3-hydroxyglutaryl nitrile to (R)-4-cyano-3-hydroxybutyric acid, the ethyl ester of which is an intermediate to the Atorvastatin [21]. Recently ethyl (*S*)-4-chloro-3-hydroxybutyrate ((*S*)-ECHB) is also suggested to be a chiral intermediate for the production of Atorvastatin [22]. (*S*)-ECHB can be synthesized through two successive enzyme reactions and a chemical reaction (Fig. 1). NHase converts racemic 4-chloro-3-hydroxybutyronitrile (CHBN) into 4-chloro-3-hydroxybutyramide (CHBamide) and then amidase converts the amide substrate into (*S*)-4-chloro-3-hydroxybutyric acid (CHBacid).

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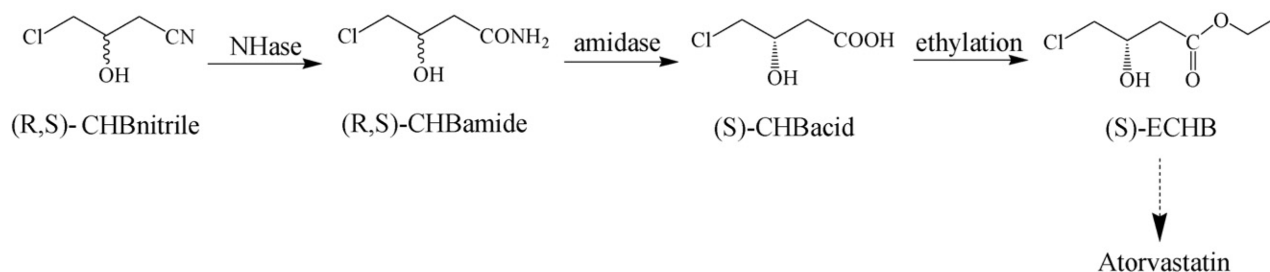

Fig. 1. Conversion of CHBN to (*S*)-ECHB. (*S*)-CHBacid can be prepared from racemic CHBN using NHase and enantioselective amidase. (*S*)-ECHB can be made from (*S*)-CHBacid through an ethylation reaction and be used for the synthesis of Atorvastatin.

As part of this research, a bacterial strain capable of converting CHBN to CHBacid was found, and a NHase enzyme was partially purified and characterized. Finally, this purified enzyme was used to produce CHBamide from CHBN.

2. Experimental

2.1. Chemicals

The CHBN, CHBamide, and CHBacid were obtained from Equispharm Co., Ltd. (Korea). Meanwhile, the various nitriles and amides were purchased from Sigma–Aldrich Inc. (St. Louis, MO), Fluka, Chemie GmbH (Buchs, Switzerland) and Junsei Chemical Co., Ltd. (Tokyo, Japan), and the DEAE-Sepharose and Phenyl-Sepharose were obtained from AmerSham BioScience AB (Uppsala, Sweden) and Bio-gel A from Bio-Rad Laboratories Inc. (Hercules, CA).

2.2. Microorganism, media and growth conditions

The bacterial strain N′4 (KCTC19425) was found to have the ability to convert CHBN. A LB medium containing 1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar was used for cultivation purposes. The strain was inoculated into an LB broth, shaken at 230 rpm, and cultivated at 30 °C for 48 h [2,13,14].

2.3. Nitrile hydratase assay

The standard NHase assay technique employed was a spectrophotometric method which made use of methacrylonitrile. Enzyme activity was assayed at 15 °C by measuring the formation of methacrylamide from methacrylonitrile (5 mM) in a 10 mM potassium phosphate buffer (pH 7.5). Methacrylamide was then detected by measuring absorbance at 224 nm [13,19]. One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 μmol of methacrylamide per minute.

The following amidase coupling method was also used to test hydratase activity toward various nitrile substrates. A reaction mixture (400 μL) containing 5 mM nitrile substrate, and an appropriate amount of purified enzymes in 10 mM potassium phosphate buffer (pH 7.5) was utilized. The NHase reaction was performed at 15 °C for 10 min and then stopped. In order to inactivate the NHase enzyme, the reaction mixture was immediately incubated for 5 min at 65 °C, and then transferred to ice. Sufficient amounts of amidase were added to each reaction mixture. An enzyme reaction at 40 °C for 1 h was brought about in order for the amidase to hydrolyze all amides into acids. The ammonia produced was determined using the Fawcett method [23]. The amidase enzyme used in this experiment was the *Rhodococcus erythropolis* No. 7 amidase expressed in *Escherichia coli* BL21 (DE3) cell [24]. The amount of nitrile substrates used in the reactions (400 μL) was 2 μmol. The amount of amidase used in the coupling reaction corresponded to 0.07 units, 0.13 units, 0.15 units, 0.26 units, 0.53 units, 1.3 units, and 0.52 units activities toward acetamide, acrylamide, methacrylamide, benzamide, butyramide, isobutyramide, and propioamide, respectively, which were sufficient activities to hydrolyze all amide products (less than 2 μmol) within 1 h at 40 °C.

One unit of NHase was defined as the amount of enzyme needed to release 1 μmol of amide (NH_3 equivalent) from the nitrile per minute.

2.4. Purification of nitrile hydratase from R. erythropolis N′4

The strain was grown in an LB medium (1 L) containing 0.1% ε-caprolactam [25–27] at 30 °C for 48 h. As the enzyme was unstable [8], all purification steps were performed in buffer A (50 mM potassium phosphate buffer pH 7.5 and 20 mM sodium butyrate) at 4 °C.

The cultured cells were resuspended in 50 mL of buffer A, and sonicated at 0 °C. The supernatant was fractionated with $(NH_4)_2SO_4$. The proteins precipitating between 30% and 60% saturation were dissolved in 5 mL of buffer A, dialyzed against the same buffer, and loaded onto a DEAE-Sepharose column. The column was washed with the same buffer, and then eluted with a linear gradient of KCl (0–500 mM) in buffer A. The active fractions were collected, and ammonium sulfate was added to 10% saturation. The solution was applied to a Phenyl-Sepharose column. The enzyme was eluted with a linear decreasing gradient of $(NH_4)_2SO_4$ (10–0%) in buffer A. The active fractions were thereafter collected, concentrated, and used for gel filtration chromatography. The enzyme solution was loaded onto a Bio-gel A column (1.5 cm × 98 cm) equilibrated with 20 mM sodium butyrate, 150 mM NaCl, 50 mM potassium phosphate buffer (pH 7.5) and then eluted with the same buffer.

Protein concentrations were determined using either a Bio-Rad Bradford protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA) [28] or by measuring absorbance at 280 nm. A SDS-PAGE was performed in 12% polyacrylamide gel using a Bio-Rad Model Mini PROTEIN 3 System. After electrophoresis, the gel was stained with Coomassie blue R-250.

2.5. Effect of temperature and pH

To determine the optimum temperature of the NHase N′4, the enzyme was, along with 5 mM methacrylonitrile, incubated at 5–50 °C for 10–20 min. Meanwhile, to determine temperature stability, the enzyme was pre-incubated at 5–50 °C for 30 min, with the remaining activity subsequently assayed at 15 °C.

The following buffers (50 mM) were used to study the effects of pH: pH 4–6, acetic acid/sodium acetate; pH 7–9, KH_2PO_4/K_2HPO_4; pH 9.5–11, K_2HPO_4/K_3PO_4. To verify pH stability, the enzyme was pre-incubated in various pH buffers at 0 °C for 30 min and then adjusted to pH 7.5. The enzyme's residual activity was then determined.

2.6. *Effect of organic acids on NHase activity*

The enzyme was pre-incubated for 30 min at 30 °C along with 20 mM of various organic acids. The residual NHase activity was then assayed by standard method.

2.7. *Inhibition profile*

To determine the effect of various chemical reagents and inhibitors on NHase N′4 activity, the enzyme was pre-incubated with chemical reagent/inhibitors (1 mM) in a 10 mM potassium phosphate buffer (pH 7.5) at 0 °C for 30 min. After incubation, 5 mM methacrylonitrile was added to each buffer, and the remaining activity was determined. In the case of 1, 10-phenanthroline, the inhibitor's effect was determined by the amidase-coupling method with acrylonitrile.

2.8. *CHBN-hydratase activity assay*

TLC and gas chromatography (GC) analysis were used to assay the conversion of CHBN with purified NHase. A reaction mixture (400 μL) containing 12.5 mg of CHBN, 4.7 μg (1.13 units) of purified enzyme in a 10 mM potassium phosphate buffer (pH 7.5) was prepared and incubated at 15 °C for 120 min. The reaction mixture (25 μL) was spotted on silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany), and developed using a mixture solvent (hexane:ethyl acetate:acetic acid = 2:3:0.5). Thereafter, the silica was heated, and the spots on each compounds were detected using a mixture reagent containing 9.2 mL *p*-anisaldehyde, 3.75 mL acetic acid, 338 mL ethanol (95%), and 12.5 mL sulfuric acid.

CHBN degradation was also quantitatively assayed through GC. A reaction mixture (800 μL) containing 25 mg of CHBN, 9.4 μg (2.26 units) of purified enzyme in a 10 mM potassium phosphate buffer (pH 7.5) was prepared and incubated at 15 °C for 120 min. The reaction mixture (100 μL) was mixed with 400 μL ethyl acetate and 50 μL saturated K_2CO_3. After centrifugation, an ethyl acetate phase was obtained and dried. Thereafter, 400 μL of methylene chloride (MC) was added. The acetylation reaction was then carried out by adding 20 μL pyridine and 10 μL acetyl chloride. A 200 μL of 1 M HCl was then added after 1 h-reaction at room temperature. The lower MC layer was recovered and 200 μL of saturated $NaHCO_3$ was then added. The MC layer was obtained and injected into the GC. The GC was carried out with an FID detector and a chirasil-dex CB DF = 0.25 (Wcot fused silica 25 m × 0.25 mm) column (Varian Inc., Lake Forest, CA) from the HP 5890 series II gas chromatography (Hewlett Packard Co., Palo Alto, CA). The initial column temperature was 70 °C with a 5 °C/min gradient performed until 180 °C, and then continued for 2 min thereafter. While the carrier gas was nitrogen, the detector temperature was 220 °C.

3. Results and discussion

3.1. *Selection of R. erythropolis N′4 strain converting CHBN to CHBacid*

Many isolated bacterial strains were tested for their conversion activity toward the CHBN molecule. Of these, bacterial strain N′4 was found to possess a strong converting activity. The strain was deposited in the Korea Type Culture Collection (KCTC 19425) for further studies. Cultured whole cells were shown to be able to convert CHBN into CHBacid (Fig. 2). This activity was dramatically induced by ε-caprolactam, a known inducer of nitrilase or NHase/amidase genes in some bacterial strains [25–27]. Conversely, benzonitrile and 2-phenyl propionitrile, which are known to induce nitrilase and NHase/amidase in other bacteria [7], failed to induce any CHBN-converting activity at all (Fig. 2).

Although whole cells converted CHBN into CHBacid, as the final products of both enzyme systems were the same compound, CHBacid, doubts emerged as to whether this conversion was mediated by nitrilase or NHase/amidase enzyme systems. CHBN was found to hydrate solely into CHBamide when this conversion reaction was performed at 15 °C (Fig. 2). However, this CHBamide was subsequently hydrolyzed into CHBacid when the reaction temperature was raised to 40 °C. These results clearly show that NHase and

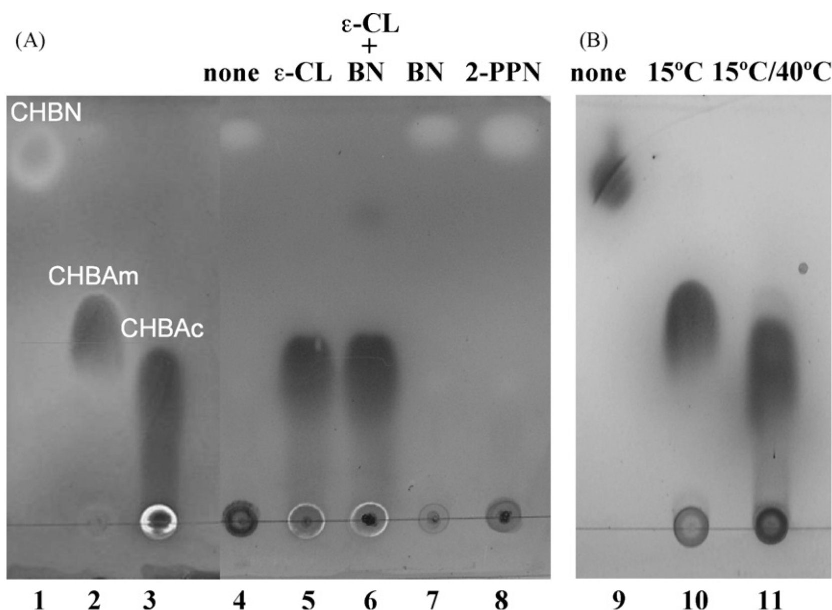

Fig. 2. Enzyme induction by ε-caprolactam. (A) *R. erythropolis* N′4 was cultured in the presence of possible inducers, and then each cultured intact cells were tested for CHBN-converting activity. Lanes 1, 2, and 3 correspond, respectively, to CHBN, CHBamide, and CHBacid. Meanwhile, Lane 4 represents no inducer; lane 5, ε-caprolactam (ε-CL); lane 6, both ε-CL and benzonitrile (BN); lane 7, BN; lane 8, 2-phenyl propionitrile (2-PPN). (B) *R. erythropolis* N′4 was cultured with ε-CL and the cultured intact cell was incubated with CHBN sequentially at 15 °C for 20 min and then at 40 °C for 20 h. Lane 9, before incubation; lane 10, after incubation at 15 °C; lane 11, after sequential incubation at 15 °C and 40 °C.

Table 1
Purification of nitrile hydratase from *R. erythropolis*

Step	Protein (mg)	Activity[a] (U)	Specific activity[a] (U/mg)	Yield (%)	Purification fold
Cell-free extract	120	1400	11	100	1
$(NH_4)_2SO_4$ Ppt.	49	1000	20	71	1.8
DEAE-Sepharose	8.6	770	90	55	8.2
Phenyl-Sepharose	0.21	180	860	13	78

[a] NHase activity was assayed using 5 mM methacrylonitrile as substrate at 15 °C.

amidase enzymes act successively on CHBN and CHBamide substrates, and also showed that their working temperatures are very different from one another.

The partial 16S rRNA sequence (578 bp) (GenBank accession No. EU130913) of the strain N′4 was found to have an identity that was 100% that of *R. erythropolis*. As such, the strain was named *R. erythropolis* N′4.

3.2. Purification of R. erythropolis N′4 NHase

In this research, the following process was used to partially purify the NHase enzyme from the *R. erythropolis* N′4 cell. After having sonicated the cells, soluble and insoluble fractions were obtained through centrifugation. The amount of NHase activity in the soluble fraction and insoluble fraction were measured at 73.8 U mL^{-1} and 31.7 U mL^{-1}, respectively. These results implied that the *R. erythropolis* NHase enzyme exists as intracellular soluble form. The NHase activity in the insoluble fraction might be due to undisrupted cell.

The cell-free extract (soluble fraction) had a NHase activity of 11 U mg^{-1} toward methacrylonitrile. The NHase enzyme was purified through $(NH_4)_2SO_4$ precipitation, DEAE-Sepharose chromatography, and Phenyl-Sepharose chromatography (Table 1). NHase was then bound to DEAE-Sepharose resin at pH 7.5 and then eluted with salt (KCl, 0–500 mM). Specific activity was 90 U mg^{-1}, which represents an eightfold increase in activity. While Phenyl-Sepharose chromatography was found to be the most effective step, specific activity increased by as much as 860 U mg^{-1}. A SDS-PAGE showed that the purified NHase had two subunits, namely α-subunit and β-subunit (Fig. 3).

On SDS-PAGE gel, the molecular weights of α and β subunit of the enzyme were estimated to be 27 and 29 kDa, respectively. Each subunit molecular weights are similar with those of most bacterial NHases reported [11]. The NHase genes have been cloned and their nucleotide sequences analyzed in this research (unpublished data). The calculated molecular weights of α and β subunit of the enzyme were 22,953 and 23,486, respectively, which were a little bit different from those obtained from SDS-PAGE experiment. This discrepancy has been observed from many other NHase enzymes including *Rhodococcus* NHases, even though the reason has not been addressed yet.

Table 2
Substrate specificity of purified NHase N′4

Substrate	Formula	Relative activity (%)[a]
Butyronitrile	$CH_3CH_2CH_2CN$	483
Propionitrile	CH_3CH_2CN	234
Acrylonitrile	$CH_2{=}CHCN$	146
Methacrylonitrile	$CH_2{=}C(CH_3)CN$	100
Isobutyronitrile	$CH_3CH(CH_3)CN$	12
Benzonitrile	C_6H_5CN	4
Acetonitrile	CH_3CN	1

[a] The amount of amide was determined by amidase-coupling reaction. After amidase activity to each amide was measured, excess amount of amidase was then used in the coupling reactions.

Gel filtration chromatography with the purified enzyme showed that its partition coefficient (K_{av}) was 0.42 and that its molecular weight was about 92.3 kDa and it consisted of four subunits of $\alpha_2\beta_2$ (Fig. 3B).

3.3. Biochemical characterization of R. erythropolis N′4 NHase

In order to test the hydratase activity of the purified NHase toward various nitrile compounds, the above-mentioned amidase-coupling method was used. In this regard, butyronitrile and propionitrile were found to hydrate more rapidly than benzonitrile, acrylonitrile and acetonitrile (Table 2). This result indicates that the enzyme prefers an aliphatic saturated nitrile substrate over an aliphatic unsaturated or aromatic one.

In order to test other biochemical properties, methacrylonitrile was used as the substrate in connection with the direct spectropho-

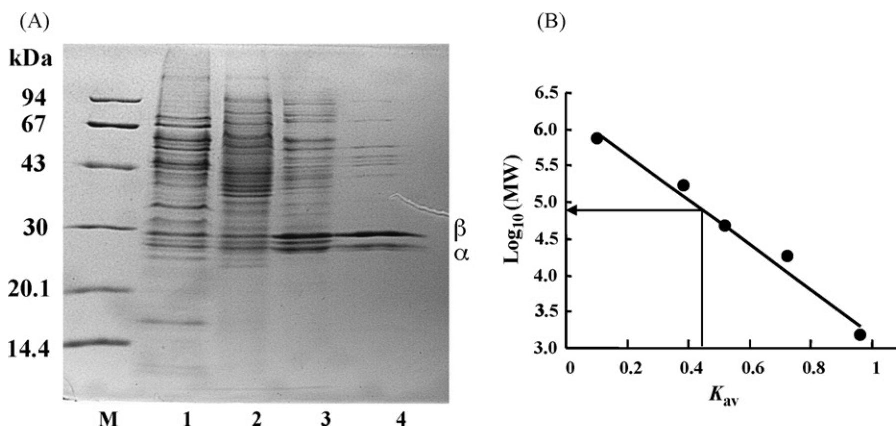

Fig. 3. SDS-PAGE and gel filtration chromatography of NHase N′4. (A) M, molecular weight markers; lane 1, cell-free extract of *R. erythropolis* N′4; lane 2, proteins after $(NH_4)_2SO_4$ fractionation (30–60%); lane 3, after DEAE-Sepharose; lane 4, after Phenyl-Sepharose. (B) Standard curve of partition coefficient (K_{av}) and log molecular weight was obtained against thyroglobulin (MW 670,000), γ-globulin (MW 158,000), ovalbumin (chicken) (MW 44,000), myoglobin (horse) (MW 17,000), vitamin B_{12} (MW 1350). The K_{av} value of NHase was 0.42.

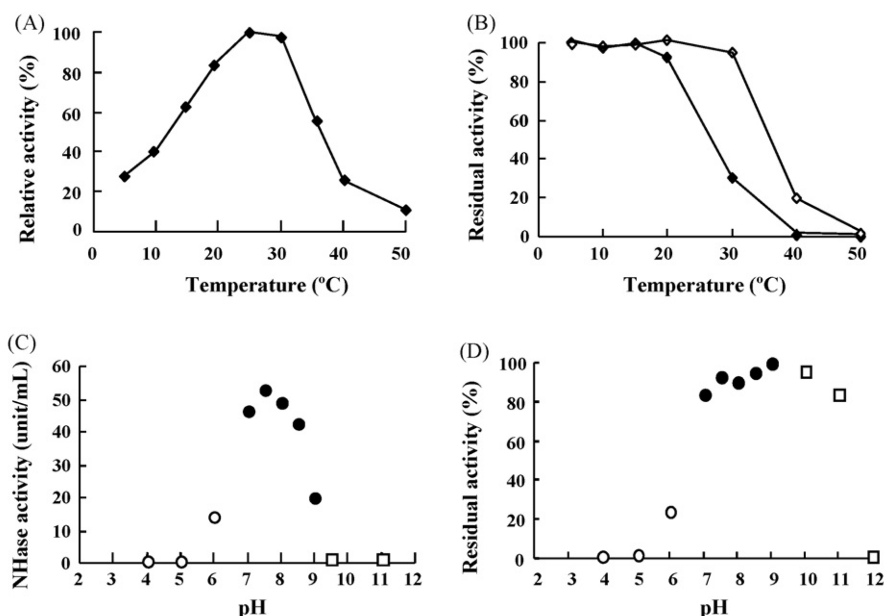

Fig. 4. The effect of temperature and pH on the activity and stability of NHase N′4. (A) The reactions were carried out at various temperatures. (B) After pre-incubation for 30 min at various temperatures in a 10 mM potassium phosphate buffer, pH 7.5 with (◊) or without (♦) 20 mM sodium butyrate, the enzyme's remaining activity was assayed at 15 °C. (C) Reactions were carried out in the following buffers (50 mM); pH 4–6, acetic acid/sodium acetate (○); pH 7–9, KH_2PO_4/K_2HPO_4 (●); pH 9.5–11, K_2HPO_4/K_3PO_4 (□). (D) After pre-incubation at 0 °C for 30 min in the following buffers (50 mM): pH 4–6, acetic acid/sodium acetate (○); pH 7–9, KH_2PO_4/K_2HPO_4 (●); pH 9.5–11, K_2HPO_4/K_3PO_4 (□), the enzyme's remaining activity was assayed at 15 °C pH 7.5.

tometric method. Purified NHase showed an optimum temperature of 25 °C, and its activity decreased rapidly at temperatures of over 30 °C (Fig. 4A). While its thermostability was very low, it was stable at 20 °C, before decreasing rapidly thereafter (Fig. 4B). That is, NHase N′4 was thermolabile with half life of 15 min and 2.6 h at 30 °C and 20 °C, respectively. In this regards, NHase N′4 is in many ways similar with several Fe-type NHase. To this end, while Fe-type NHase have in general been thermolabile, the Co-type NHases have for the most part been relatively thermostable [8–10,13].

An optimum pH of 7.5 was recorded (Fig. 4C), with stability reached at pH 7–11 (Fig. 4D).

The thermostability of Fe-type NHase has been known to increase in the presence of various organic acids [8]. As part of this research, five different organic acids were added to the enzyme during incubation at 30 °C. To this end, although pyruvate had little effect, and lactate was even found to have a destabilizing effect, butyrate, valerate, and caproate all produced stabilizing effects (Fig. 5A). The stabilization effects of those last three organic acids reached the maximum value over 10 mM concentration (Fig. 5B). The thermostability of the Fe-type NHase increased by 10 °C when butyrate was present (Fig. 4B). While the effect was obvious, the detailed molecular mechanism remained unknown. To rectify this situation we added butyrate to the enzyme storage buffer and to the buffers used during enzyme purification.

The results found in Table 3 revealed that NHase was inhibited to a remarkable extent by various sulfhydryl reagents, oxidizing reagents, and PMSF. Moreover metal chelating reagents and metal ions were found to moderately inhibit the NHase. These results imply that the active site of the enzyme consists of cysteine, serine, and metal ions.

The NHase gene of this strain (N′4) was cloned and sequenced as part of efforts to elucidate the active site residue. This residue was subsequently determined to consist of two open reading frames encoding α and β subunits (GenBank accession Nos. EU130914 and EU130915, respectively). The protein sequences of these two

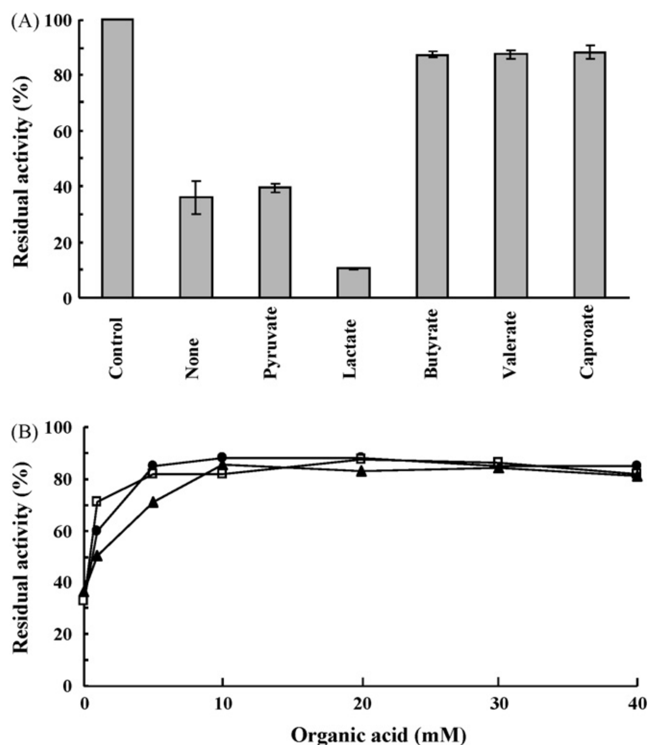

Fig. 5. Effects of organic acids on NHase N′4 stability. (A) The enzyme was incubated for 30 min at 30 °C in a 10 mM potassium phosphate buffer (pH 7.5) containing various organic acids (20 mM), with NHase activity thereafter assayed by spectrophotometric assay. The remaining activity was compared with the enzyme that had not been pre-incubated (control). (B) The enzyme was incubated for 30 min at 30 °C with various concentrations of organic acids (▲, butyrate; ●, valerate; □, caproate), and then the NHase activity was assayed under the standard condition.

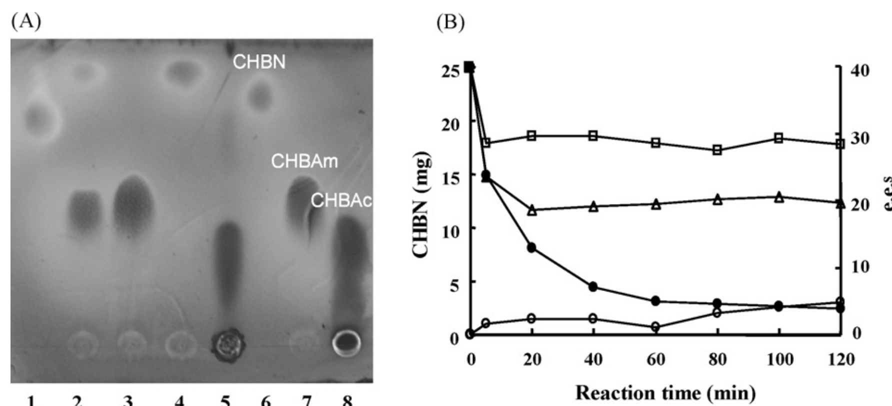

Fig. 6. CHBN-hydratase activity of purified NHase N′4. (A) CHBN conversion by purified NHase was analyzed using TLC. Lanes 1, 2, and 3 were, respectively, compounds in 0 min, 10 min, and 120 min reaction mixtures; lane 4, compounds in a 120 min reaction mixture containing the enzyme inhibitor H_2O_2; lane 5, compounds in amidase-coupling reaction, and lanes 6–8, CHBN, CHBamide, and CHBacid, respectively. (B) CHBN degradation with purified NHase was analyzed using GC and a chirasil column. The amount of CHBNs in the reaction at 15 °C (●), 20 °C (△), and 30 °C (□) were measured and the ee_s values in the reaction at 15 °C were shown (○).

subunits were most similar with those of *R. erythropolis* NHase. That is, its α and β subunits had identities of 100% and 99.1% with α (AAP57640) and β (AAP57641) subunits of *R. erythropolis* NHase, respectively. The crystal structure of *R. erythropolis* NHase, which has an unique non-heme iron active center consisting of cysteine, cyteine-sulfinic acid (Cys-SO_2H), cysteine-sulfenic acid (Cys-SOH), and serine residues [10], has already been elucidated. The post-translational modifications of these two cysteine residues are known to be very important in terms of NHase activity [10,29–31]. Accordingly, NHase N′4 seemed to share the same non-heme iron active site, and that this active site consists of Cys109, Cys112, Ser113, and Cys114.

Most NHase have an unique signature sequence of Cys-Xxx-Leu-Cys-Ser-Cys, which is directly related with the above active site [10,11,17]. Fe-type NHases have Ser amino acid at the Xxx residue, whereas Co-type NHases have Thr amino acid at that site. Sequence analysis showed that NHase N′4 had Cys^{109}-Ser^{110}-Leu^{111}-Cys^{112}-Ser^{113}-Cys^{114}, suggesting again that it be a Fe-type enzyme.

On the other hand, the fact that post-translational modification of the two cysteine residues represents a pre-requisite for enzyme activity corresponds well with the results of our own experiments showing that NHase N′4 is inhibited by many chemical compounds, including sufhydryl reagents and oxidizing reagents.

Table 3
Effect of inhibitors on NHase N′4 activity

Reagent (1 mM)	NHase activity (%)
None	100
Sulfhydryl reagents	
Copper sulphate	5
Iodoacetamide	78
Silver nitrate	18
Mercury chloride	11
Chelating reagents	
EDTA	85
1,10-Phenanthroline[a]	61
Serine protease inhibitor	
PMSF	17
Reducing reagent	
Dithiothreitol	82
2-Mercaptoethanol	91
Oxidizing reagent	
Hydrogen peroxide	3
Ammonium persulphate	48
Metals	
Ferrous sulphate	46
Zinc chloride	44
Cobalt chloride	80

NHase was pre-incubated at 0 °C for 30 min with various reagents. Remaining NHase activities were measured with methacrylonitrile substrate.

[a] Remaining NHase activity was measured by amidase-coupling reaction with 5 mM acrylonitrile. Residual activity of amidase for 1 mM 1,10-phenanthroline was already determined to be 92%.

3.4. CHBN-hydratase activity of purified NHase N′4

Based on molecular composition (α, β subunits), protein sequence, and substrate specificity, the NHase N′4 purified from *R. erythropolis* N′4 as part of this research appears to be a typical Fe-NHase enzyme. A TLC analysis revealed that this purified NHase N′4 is capable of converting CHBN into CHBamide with time course (Fig. 6A, lanes 1–3). However, no CHBamide was produced when a strong inhibitor, H_2O_2, was added (Fig. 6A, lane 4). Moreover, all CHBamide was converted to CHBacid when excess amidase enzyme was added (Fig. 6A, lane 5).

A GC analysis showed that the amount of CHBN in the reaction mixture was decreased rapidly by the purified NHase at 15 °C (Fig. 6B). Under this condition, the initial conversion rate of the reaction was 215 mg CHBN consumed min^{-1} mg^{-1}. But under 20 °C and 30 °C reaction conditions, CHBN was degraded only by 50% and 28%, respectively. At 15 °C, although enantioselectivity was not observed toward racemic CHBN (Fig. 6B), the NHase N′4 enzyme efficiently converted CHBN into CHBamide. In fact, *R. erythropolis* amidase used in this coupling assay experiment had some enantioselectivity, although it was low for direct commercial use; an ee value toward CHBamide was 52% when the conversion yield was 57% [24]. Its enantioselectivity could be improved by a further research using molecular evolution technique.

Taken together, these demonstrate that when used in conjunction with a suitable amidase, this enzyme can be utilized to synthesize the intermediate of a hypercholesterolemia drug, namely ethyl (*S*)-4-chloro-3-hydroxybutyrate (Fig. 1).

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