Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

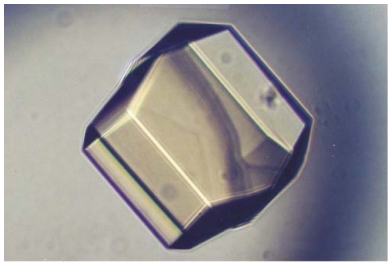
Cloning, purification, crystallization and preliminary X-ray diffraction analysis of nitrile hydratase from the themophilic *Bacillus smithii* SC-J05-1

Shinji Hourai,[a]* Takeshi Ishii,[b] Misao Miki,[a] Yoshiki Takashima,[b] Satoshi Mitsuda[b] and Kazunori Yanagi[a]

[a]Sumitomo Chemical Co. Ltd Environmental Health Science Laboratory, 3-1-98 Kasugade-naka, Konohanaku, Osaka 554-8558, Japan, and [b]Sumitomo Chemical Co. Ltd Agricultural Chemicals Research Laboratory, 4-2-1 Takatsukasa, Takarazuka, Hyogo 665-8555, Japan

Correspondence e-mail: shinji-horai@ds-pharma.co.jp

Received 10 August 2005
Accepted 27 September 2005
Online 20 October 2005



Nitrile hydratase (NHase) converts nitriles to the corresponding amides and is recognized as having important industrial applications. Purification, cloning, crystallization and initial crystallographic studies of the NHase from *Bacillus smithii* SC-J05-1 (Bs NHase) were conducted to analyze the activity, specificity and thermal stability of this hydrolytic enzyme. Bs NHase was purified to homogeneity from microbial cells of *B. smithii* SC-J05-1 and the nucleotide sequences of both the α- and β-subunits were determined. Purified Bs NHase was used for crystallization and several crystal forms were obtained by the vapour-diffusion method. Microseeding and the addition of magnesium ions were essential components to obtain crystals suitable for X-ray diffraction analysis.

1. Introduction

Nitrile hydratase (NHase) converts nitriles to the corresponding amides (Yamada & Kobayashi, 1996; Kobayashi & Shimizu, 1998). Since the first discovery of an NHase in an *Arthrobacter* bacterium, several related enzymes have been found in other microorganisms (Asano *et al.*, 1982; Endo & Watanabe, 1989; Nagasawa *et al.*, 1987, 1991; Tani *et al.*, 1989; Hjort *et al.*, 1990). *Rhodococcus*, *Pseudomonas* and *Brevibacterium* are major NHase-producing microorganisms and the NHases are regarded as participating in the metabolism of nitrile compounds. Many studies of these NHases have been conducted and some of the enzymes have been used as important biocatalysts for the industrial production of acrylamide and nicotinamide (Ashina & Suto, 1993; Hwang & Chang, 1989; Nagasawa & Yamada, 1989). The NHases are composed of α- and β-subunits and can be divided into two groups according to the identity of their prosthetic metal, which can be either cobalt or iron (Kobayashi *et al.*, 1991; Nishiyama *et al.*, 1991).

In a previous report, we described the discovery of the *Bacillus smithii* strain SC-J05-1 that possesses amide-forming ability through the screening of an in-house collection of moderate thermophiles isolated from soils. We also reported studies of some of the characteristics of the NHase purified from microbial cells of *B. smithii* SC-J05-1 (Takashima *et al.*, 1998). The NHase from *B. smithii* SC-J05-1 (Bs NHase) was comprised of two different subunits, the α- and β-subunits, and contained cobalt ion as the prosthetic metal. Bs NHase catalyzed the hydration of nitriles to amides at an optimal temperature of 313 K and was stable up to 323 K. Bs NHase had wide substrate specificity and aliphatic nitriles such as acetonitrile, acrylonitrile and butyronitrile were particularly good substrates.

In this paper, we focus on the purification, cloning and crystallization of Bs NHase. The crystal structure of Bs NHase has already been solved to provide information for understanding its substrate specificity and thermal stability (Hourai *et al.*, 2003).

2. Materials and methods

2.1. Purification and N-terminal amino-acid sequence determination of Bs NHase

B. smithii SC-J05-1, which was used as the DNA source, was isolated from a soil sample and cultivated aerobically and a 20%

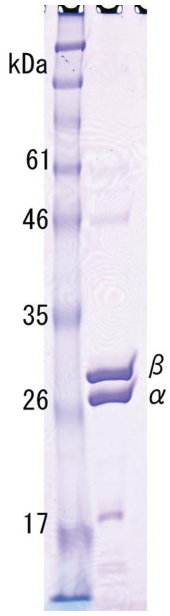


Figure 1
SDS–PAGE analysis of Bs NHase eluted from a Mono Q Sepharose column.

ammonium sulfate precipitation was conducted from a cell-free extract (Takashima *et al.*, 1998). The subsequent purification was performed using a Phenyl Sepharose HP column pre-equilibrated with 1.5 *M* ammonium sulfate. Elution was conducted with a linear gradient of 1.5–0.0 *M* ammonium sulfate in 50 m*M* potassium phosphate buffer pH 7.0. The eluted Bs NHase was then desalted and applied onto an ion-exchange Mono Q Sepharose column pre-equilibrated with 20 m*M* Bis-Tris propane buffer pH 7.0. Elution was completed with a linear gradient of 0–1.0 *M* NaCl in 20 m*M* Bis-Tris propane buffer pH 7.0.

The subunits of Bs NHase were separated on a YMC-Pack Protein RP reversed-phase column. The column was eluted with an increasing gradient of 30–60% acetonitrile in 0.1% trifluoroacetic acid aqueous solution. The N-terminal amino-acid sequences of the two subunits of Bs NHase were determined using an Applied Biosystems model 470 gas-phase sequencer (Perkin–Elmer Japan).

2.2. Cloning and nucleotide-sequence determination of the Bs NHase genes

Chromosomal DNA of *B. smithii* SC-J05-1 was prepared by the method of Saito & Miura (1963). After digestion with the restriction endonuclease *Sau*3AI, the DNA was size-selected by agarose-gel electrophoresis and purified. The resulting DNA fragments were ligated using λ ZAPII (Stratagene, La Jolla, CA, USA) as the cloning vector and packaged in λ phages. The degenerated oligonucleotides based on the amino-acid sequence of the β-subunit of the NHase (Probe-A) and the insert DNA of Probe-B described in §3 were used for the hybridization. These oligonucleotides were labelled using a Megalabel kit (Takara Shuzo, Kyoto, Japan) and a Ready Go

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Bs          1:MAIEQKLMDDHHEV-DPRFPHHHPRPQSFWEARAKALESLLIEKRLLSSDAIERVIKHYE 59
Pt          1:MTENI-LRKSDEEIQ------------KEITARVKALESMLIEQGILTTSMIDRMAEIYE 47
Rs          1:MSVTIDHTTENAAPAQA----------PVSDRAWALFRALDGKGLVP-DGYVEGWKKTFE 49

Bs         60:HELGPMNGAKVVAKAWTDPEFKQRLLEDPETVLRELGYFGLQGEHIRVVENTDTVHNVVV 119
Pt         48:NEVGPHLGAKVVVKAWTDPEFKKRLLADGTEACKELGIGGLQGEDMMWVENTDEVHHVVV 107
Rs         50:EDFSPRRGAELVARAWTDPEFRQLLLTDGTAAVAQYGYLGPQGEYIVAVEDTPTLKNVIV 109

Bs        120:CTLCSCYPWPLLGLPPSWYKEPAYRSRVVKEPRKVLQEFG-LDLPDSVEIRVWDSSSEVR 178
Pt        108:CTLCSCYPWPVLGLPPNWFKEPQYRSRVVREPRQLLKEEFGFEVPPSKEIKVWDSSSEMR 167
Rs        110:CSLCSCTAWPILGLPPTWYKSFEYRARVVREPRKVLSEMG-TEIASDIEIRVYDTTAETR 168

Bs        179:FMVLPQRPEGTEGMTEEELAQIVTRDSMIGVAKVQPPKVIQE                   220
Pt        168:FVVLPQRPAGTDGWSEEELATLVTRESMIGVEPAKAV-----                   204
Rs        169:YMVLPQRPAGTEGWSQEQLQEIVTKDCLIGVAIPQVPTV---                   207
                                    (a)
Bs          1:MNGIHDVGGMDGFGKIMYVK-EEEDTYFKHDWERLTFGLVAGCMAQGLGMKAFDEFRIGI 59
Pt          1:MNGVYDVGGTDGLGPINRPA---DEPVFRAEWEKVAFAMFPATF-RAGFMG-LDEFRFGI 55
Rs          1:MDGVHDLAGVQGFGKVPHTVNADIGPTFHAEWEHLPYSLMFAGVAELGA-FSVDEVRYVV 59

Bs         60:EKMRPVDYLTSSYYGHWIATVAYNLLETGVLDEKELEDRTQAFMEKPDTKIQR-WENPKL 118
Pt         56:EQMNPAEYLESPYYWHWIRTYIHHGVRTGKIDLEELERRTQYYRENPDAPLPEHEQKPEL 115
Rs         60:ERMEPRHYMMTPYYERYVIGVATLMVEKGILTQDELESLAG------------------- 100

Bs        119:VKVVEKALLEGLSPVREVSSFPRFEVGERIKTRNIHPTGHTRFPRYVRDKYGVIEEVY-G 178
Pt        116:IEFVNQAVYGGLPASREVDRPPKFKEGDVVRFSTASPKGHARRARYVRGKTGTVVKHH-G 175
Rs        101:-GPFPLSRPSESEGRPAPVETTTFEVGQRVRVRDEYVPGHIRMPAYCRGRVGTISHRTTE 159

Bs        178:AHVFPDDAAHRKG-ENPQYLYRVRFDAEELWGV--KQNDSVYIDLWEGYLEPVSH      229
Pt        175:AYIYPDTAGNGLG-ECPEHLYTVRFTAQELWGPEGDPNSSVYYDCWEPYIELVDT      228
Rs        160:KWPFPDAIGHGRNDAGEEPTYHVKFAAEELFGS-DTDGGSVVVDLFEGYLEPAA-      212
                                    (b)
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Figure 2
Amino-acid sequence alignments of (*a*) the α-subunits and (*b*) the β-subunits of NHases from *B. smithii* SC-J05-1 (Bs), *P. thermophila* (Pt) and *Rodococcus* sp. N-771 (Rs). The determined N-terminal amino-acid sequences of the α- and β-subunits of Bs NHase are underlined. Residues in the characteristic cobalt- or iron-binding motifs are indicated by bold characters.

Random Labelling Kit (Amarsham Pharmacia Biotech, Tokyo, Japan), respectively. The library was screened by a plaque-hybridization method and the nucleotide sequence was determined by the deoxy chain-termination method.

2.3. Dynamic light-scattering measurement and crystallization of Bs NHase

The purified Bs NHase obtained from a Mono Q Sepharose column was used for dynamic light-scattering measurements and crystallization. Dynamic light-scattering measurements were performed using Dyna Pro MS/X (Protein Solutions). Bs NHase (1 mg ml^{-1}) was prepared in solutions of 20 m*M* Bis-Tris propane buffer pH 8.0 and filtered through 0.1 µm glass filters before measurements, which were performed at 293 K.

Purified Bs NHase was dialyzed into 20 m*M* Bis-Tris propane buffer pH 8.0 and concentrated to 10 mg ml^{-1}. The NHase was then crystallized using the hanging-drop method. Drops consisting of 1 µl enzyme solution mixed with 1 µl reservoir solution were equilibrated against 500 µl reservoir solution at 293 K. The Crystal Screen I and Screen II (Hampton Research) sets of screening conditions were used for the first screening. The optimization of crystallization conditions was performed by altering the pH, PEG concentration and additives and by using a microseeding method.

2.4. X-ray data collection

The crystals were mounted in glass capillaries and X-ray diffraction data were collected at room temperature on a Rigaku R-AXIS IIc imaging-plate system using Cu $K\alpha$ radiation from a Rigaku RU300 rotating-anode generator operated at 40 kV and 100 mA. The crystal-to-detector distance was 120 mm and the oscillation range was set to 1.5°. The image data were processed using the *Crystal Clear* program (Rigaku).

3. Results and discussion

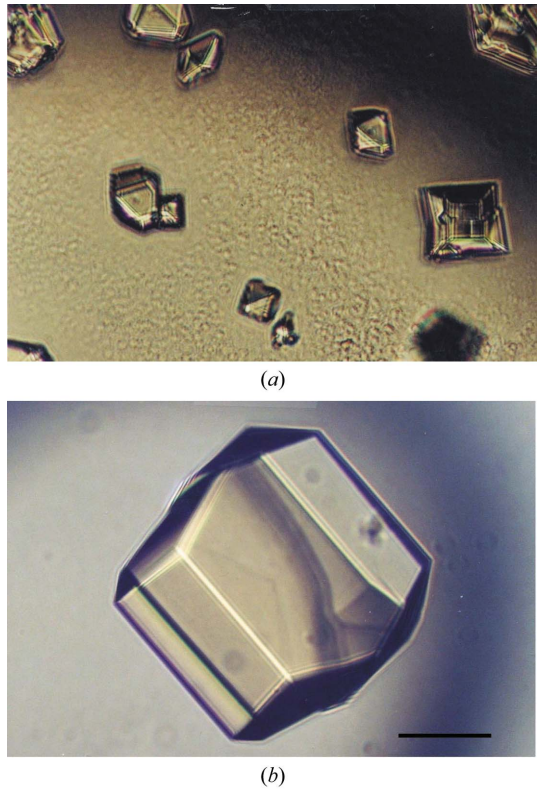
3.1. Cloning and sequence determination of the Bs NHase genes

The purity of Bs NHase was estimated using SDS–PAGE and was found to be almost homogeneous (Fig. 1). The N-terminal amino-acid sequences of the α- and β-subunits of Bs NHase were determined (Fig. 2). A probe for the degenerated oligonucleotides (Probe-A) based on the N-terminal amino-acid sequence of the β-subunit (5′-ATGGATGGITTTGGIAAAATTATGTATGTIAAAGAAGAAG-AAGATACITATTTTAAACATGATTGGGA-3′; I indicates inosine) was designed and synthesized for hybridization. Chromosomal DNA isolated from *B. smithii* SC-J05-1 was partially digested with the restriction endonuclease *Sau*3AI, size-selected from 4 to 7 kbp on agarose gel and a λ ZAPII library was constructed containing the digested genomic DNA. The gene library was screened with Probe-A and a positively hybridizing phage clone was isolated. An insert of about 1.9 kbp was converted to a pBluescript plasmid. The gene library was screened again using the 1.9 kbp insert as the probe (Probe-B) and the complete sequence was determined. Two open reading frames were identified in the region from the sequence analysis, one coding for the α-subunit comprising 220 amino acids (M_r = 25 378) and the other coding for the β-subunit comprising 229 amino acids (M_r = 26 662). The deduced molecular weights of the α- and β-subunits of Bs NHase were 25.4 and 26.7 kDa, respectively, which were similar to those estimated by SDS–PAGE (Fig. 1). The deduced N-terminal amino-acid sequences of the α- and β-subunits of Bs NHase were identical to those determined by N-terminal sequence analysis (Fig. 2). The full nucleotide sequences of the α- and β-subunits of Bs NHase have been deposited in the EMBL database (accession Nos. E13931 and E13932, respectively).

Sequence analysis of the amino-acid sequence of the Bs NHase α-subunit showed it to have 83.0% identity with the NHase α-subunit from *Bacillus* sp. BR449 (Kim & Oriel, 2000). Likewise, the amino-acid sequence of the Bs NHase β-subunit showed 81.0% identity with the NHase β-subunit from *Bacillus* sp. BR449. The amino-acid sequence identity between Bs NHase and the NHase from *Rodococcus* sp. N-771 (Nagashima *et al.*, 1998) was 53.2% for the α-subunit and 33.9% for the β-subunit. The amino-acid sequence identity between Bs NHase and the NHase from *Pseudonocardia thermophila* (Miyanaga *et al.*, 2001) was 64.0% for the α-subunit and 42.2% for the β-subunit (Fig. 2). In particular, the NHase α-subunit was found to contain the cobalt-binding motif CTLCSC.

3.2. Dynamic light-scattering measurement and crystallization

The dynamic light-scattering measurements gave a molecular weight of 110 kDa, suggesting that Bs NHase forms an $\alpha_2\beta_2$ hetero-tetramer. This result is consistent with that of gel-filtration experiments (Takashima *et al.*, 1998). During screening of crystallization conditions, several crystal forms were obtained. Of these, we refined crystallization condition No. 23 from Crystal Screen I (30% polyethylene glycol 400 and 0.2 *M* magnesium chloride in 0.1 *M* HEPES at pH 7.5) and found that the microseeding method and the addition of magnesium ion were essential components for obtaining crystals suitable for X-ray analysis. Microseeding was performed using seeds of small crushed crystals grown against a reservoir solution containing 32% polyethylene glycol 400 and 0.2 *M* magnesium chloride in 0.1 *M* HEPES pH 6.8 (Fig. 3*a*). Seeds from serial dilutions



(*a*)

(*b*)

Figure 3
Crystals of Bs NHase. (*a*) Small crystals obtained by initial screening used for seeds. (*b*) Single crystal obtained by the microseeding method. The scale bar represents 0.1 mm.

were introduced into freshly made drops. The crystals used for data collection were grown against a reservoir solution containing 30% polyethylene glycol 400 and 0.2 *M* magnesium chloride in 0.1 *M* HEPES pH 7.5. The crystals grew to maximum dimensions of approximately 0.3 × 0.3 × 0.2 mm in one month under the optimum growth conditions (Fig. 3*b*).

3.3. X-ray data analysis

The X-ray data show that crystals of Bs NHase are tetragonal, space group *I*422. Details of the data have been presented previously in the report of the structure determination (Hourai *et al.*, 2003).

We have reported here the purification, cloning and crystallization of Bs NHase, which has led to determination of the structure. Microseeding and the addition of magnesium ion were essential for the optimization of crystal size and quality. The crystal structure of Bs NHase (PDB 1v29; Hourai *et al.*, 2003) allowed us to design mutant NHases from *B. smithii* SC-J05-1 for biophysical analysis.

We thank Dr Henrik Jorgensen of Sumitomo Chemical Co. Ltd for his comments and corrections to the manuscript.

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