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Discovery of a new Fe-type nitrile hydratase efficiently hydrating aliphatic and aromatic nitriles by genome mining

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ABSTRACT

Microbial nitrile hydratases (NHases) are important industrial catalysts to produce valuable amides. However, only some NHase genes have been reported and studied at the molecular level. In this study, we developed a genome mining method to discover Fe-type NHases from GenBank. The putative NHase gene from *Pseudomonas putida* F1 was cloned and functionally expressed in *Escherichia coli* BL21 (DE3) by assisting of a putative activator gene adjacent to β-subunit region. Three recombinant plasmids containing NHase gene or the activator gene were designed and constructed. Maximal enzyme activity was obtained when the structural and activator genes were transcribed as one unit in plasmid pCDFDuet-1 at 18 °C. However, the expressed product did not show any NHase activity when the downstream activator gene was ignored, and the product completely existed in insoluble inclusion body. The activator gene might be involved in protein folding of the α- and β-subunits of NHase. In addition, the Fe-type NHase exhibited broad substrate specificity. The enzyme can efficiently hydrate aromatic nitriles, such as 3-cyanopyridine, 4-cyanopyridine, and benzonitrile, asides from aliphatic nitriles preferentially. Therefore, the recombinant NHase shows potential applications in some amides preparation.

1. Introduction

Nitriles are manufactured and extensively used as starting materials or synthetic intermediates to produce fine chemicals because of their simple preparation and versatile transformation. Hydration of nitriles to amides is therefore an important reaction in the chemical and pharmaceutical industries. However, such an application is restricted because of the harsh conditions involved, such as the need for strong acids or bases at a high temperature [1]. By contrast, biotransformation of nitriles to corresponding amides is of great interest because of its mild conditions and high conversion. Nitrile hydratase (NHase, EC 4.2.1.84), which can effectively catalyze the hydration reaction, has been successfully used in the industrial production of acrylamide from acrylonitrile [2]. The enzyme is also a key enzyme in the bi-enzymatic pathway of nitrile degradation for bioremediation [3].

NHase consists of α- and β-subunits with a molecular weight region of approximately 20–28 kDa for each subunit, and usually exists as αβ dimers or $\alpha_2\beta_2$ tetramers. The both subunits are unrelated in amino acid sequences. The active site containing non-heme iron or non-corrin cobalt is buried at the interface of these two subunits [4]. Therefore, NHase can be classified into two broad groups on the basis of the metal ion component: Fe type and Co type. These two groups exhibit different enzymatic properties, particularly substrate specificity. Fe-type NHases preferentially hydrate small aliphatic nitriles, whereas Co-type NHases exhibit a high affinity for aromatic nitriles [5]. In addition, Fe-type NHase shows a unique photoreactivity regulated by an endogenous nitric oxide. The activity of Fe-type NHase is lost during aerobic incubation in the dark, but it is completely recovered upon light irradiation [6]. This special characteristic of Fe-type NHase also implies that the enzyme has potential applications in other fields, such as in biosensors or biological switches.

Many microbes exhibit NHase activity, such as *Rhodococcus*, *Pseudonocardia*, *Brevibacterium*, *Nocardia*, *Bacillus*, *Pseudomonas*, *Mezorhizobium*, *Comamonas*, and *Rhodopseudomonas*. However, these microbes can also transform amides further into carboxylic acids by their amidase, which is usually expressed along with NHase in the same operon [7]. Consequently, the yield and purity of the target product are reduced. Recombinant expression of NHase appears to be an alternative solution to this problem. Moreover, recombinant NHase is conveniently tailored and improved by protein engineering. However, previous attempts to produce NHase in *E. coli* were not successful because of the formation of inactive and insoluble protein [8,9]. To solve this problem, the incubation

Abbreviations: NHase, nitrile hydratase; ORFs, open reading frames; OD, optical density; IPTG, isopropyl-β-D-thiogalactopyranoside; DMSO, dimethylsulfoxide; PPB, potassium phosphate buffer.

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temperature was reduced to lower the growth rate of the host cells and obtain bioactive recombinant NHases in *E. coli* cytoplasm [10]. *Pichia pastoris* was also used to express NHase, but the recombinant NHase activity was quite low and unstable [11]. In addition, some elements in the NHase gene cluster were also determined to control the active expression of recombinant NHase as transcriptional regulators, particularly the NHase activator gene [12,13]. However, the co-expression of activator is not always necessary for the active expression of recombinant NHases in *E. coli*, such as NHases from *Bacillus* sp. BR449 and *Comamonas testosteroni* Ni1 [14,15]. So far, only a dozen Fe-type NHase genes have been published for cloning and functional expression in heterologous cells. Most of them were from *Rhodococcus erythropolis*, and the protein sequences shared high homology, >95% identity. Therefore, how to efficiently exploit new NHase genes and functionally express them in genetic hosts remains an important research subject.

In this post-genomic era, intensive efforts in genome sequencing are producing large amount of digital data. As of June 2013, 4435 sequencing projects of genomes have been completed, including 193 of archaea, 4056 of bacteria, and 186 of eukaryotes. And 22580 projects are in progress (http://www.genomesonline.org/). Moreover, over 152 million sequence records containing about 165 billion bases as of June 2013 were reported in the GenBank Database (http://www.ncbi.nlm.nih.gov/genbank). Mining these genetic databases to real enzymes has been gained extensive attentions. This strategy has been applied to efficiently discover different novel enzyme catalysts, such as (*R*)-selective amine transaminases, 2-naphthoic acid monooxygenases, nitrilases, carbonyl reductases, and so on [16–19]. To date, there are still no any study reports on the NHase discovery by genome mining.

In this study, we developed an approach to find new Fe-type NHases by mining all available sequence databases provided by the National Center for Biotechnology Information (NCBI) using the Fe-type NHase specific sequence motif. We found that NHase gene existed in a large number of microbial genomes, and Pseudomonas is another main cluster besides Rhodococcus. Thus, the putative NHase from *P. putida* F1 was further studied in this work. Based on the characteristics of the gene cluster of *P. putida* F1, the NHase gene was cloned and functionally expressed in *E. coli* BL21 (DE3). The recombinant Fe-type NHase exhibits broader substrate specificity compared with most reported Fe-type NHases. It can effectively hydrate some aromatic nitriles asides from aliphatic nitriles preferentially.

2. Materials and methods

2.1. Genome mining of Fe-type NHases

The general methodology for mining Fe-type NHases was shown in Fig. S1. This method consists of five main sections. Firstly, through sequence alignments of all experimentally confirmed Fe-type NHases using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), a conserved motif in α-subunit was selected as probe, KNVIVCSLCSCTAWPILGLPPTWYKSFEYRARVVREPR. This probe included the ion-binding motif, -CSLCSC-, and located in the active cavity formed at the interface between the α- and β-subunits (Fig. S2). Then, a protein BLAST was implemented to find and assemble homologous protein sequences in GenBank. The all selected putative NHase genes were from microbial genomes. The identities of these sequences were analyzed by ClustalW2, and the rates of molecular evolution were estimated by Molecular Evolutionary Genetics Analysis (MEGA) software. Based on these analyses, some target gene sequences would be cloned from microbial genome by polymerase chain reaction (PCR) to construct recombinant plasmids, and further expressed and characterized in *E. coli*.

2.2. Strains, plasmids, enzymes, and chemicals

P. putida F1 (ATCC 700007) was used as the NHase gene donor. *E. coli* DH5α (Stratagene, USA) and BL21 (DE3) (Novagen, USA) served as hosts for genetic cloning and expression. Plasmids pMD 18-T (Takara Biotechnology Dalian, China) and pCDFDuet-1 (Novagen™, Merck KGaA, Germany) were used as cloning and expression vectors, respectively. Restriction endonucleases and T4 DNA ligase were purchased from Fermentas (ThermoFisher, USA), and Ex Taq™ DNA polymerase was obtained from Takara. 3-Cyanopyridine, 4-cyanopyridine, benzonitrile, benzeneacetonitrile, acrylonitrile, isobutyronitrile, 4-chlorobutyronitrile, valeronitrile and 3-methylbenzonitrile (Aladdin Chemistry, China) and nicotinic acid and nicotinamide (Sigma–Aldrich, USA) were used. All other chemicals were of analytical grade and obtained from local commercial sources.

2.3. Media and culture conditions

E. coli strains were cultivated in Luria–Bertani (LB) media with appropriate antibiotic (100 μg/mL ampicillin or 50 μg/mL streptomycin) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 100 μM) as required. Unless otherwise stated, all cultures underwent shaking (200 rpm) at 37 °C. *P. putida* F1 was cultivated with shaking at 30 °C in liquid nutrient broth (pH 7.4) of 10 g/L peptone, 3 g/L beef extract, and 5 g/L NaCl.

2.4. DNA extraction, PCR primers design and plasmid construction

Total genomic DNA from *P. putida* F1 was extracted with a soil DNA isolation kit (E.Z.N.A.™, OMEGA). The PCR primers used in this study are shown in Table 1. Alpha F/Beta R was used to clone the structural gene of NHase. Alpha F/P47K R was utilized to obtain the entire fragment that contains the structural gene of NHase and the downstream putative activator gene (as P47K), and P47K F/P47K R was used to obtain that for the downstream activator gene. The PCRs included preliminary denaturation 95 °C for 5 min, followed by 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 57 °C or 60 °C, and 2 min extension at 72 °C. A final extension was conducted at 72 °C for 10 min. The PCR products were cloned into pMD 18-T, and then transformed into *E. coli* DH5α. Positive clones were further confirmed by gene sequencing (Shanghai Sangon, China). The identified gene fragments were digested with EcoRI/HindIII, EcoRI/XhoI, or BglII/XhoI, re-cloned into the expression vector pCDFDuet-1 with the same restricted digestion, and finally transformed into *E. coli* BL21 (DE3). These resulting plasmids were labeled pNHP1, pNHP2, and pNHP3.

2.5. Expression and purification of the recombinant NHase_F1

E. coli BL21 (DE3) transformed with pNHP1, pNHP2 and pNHP3 was grown in 100 mL LB media supplemented with streptomycin. After 2 h culture (OD_{600} = 0.8), 20 μl 0.5 M IPTG stock solution was added to induce NHase expression at 18 °C. Cultivation was continued for 12 h, and the cells were harvested by centrifugation

Table 1
Primers design and sequences including restriction sites.

Primer	Sequence[a]	Restriction site
Alpha F	5′-G*GAATTCG***ATGACGGCAACTTCAACCC**-3′	*EcoRI*
Beta R	5′-CCC*AAGCTT***TCATGCGCGCACCACTC**-3′	*HindIII*
P47K F	5′-GA*AGATCT***CATGAGTGCCGGCGCCCAG**-3′	*BglII*
P47K R	5′-CG*CTCGAG***CTAGAAAACGGCATCAGC**-3′	*XhoI*

[a] The gene specific sequences are underlined in bold and the restriction sites are in italics.