Strategy for successful expression of the 
Pseudomonas putida nitrile hydratase activator P14K in Escherichia coli

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Abstract

Background: Activators of Nitrile hydratase (NHase) are essential for functional NHase biosynthesis. However, the activator P14K in P. putida is difficult to heterogeneously express, which retards the clarification of the mechanism of P14K involved in the maturation of NHase. Although a strep tag containing P14K (strep-P14K) was over-expressed, its low expression level and low stability affect the further analysis.

Results: We successfully expressed P14K through genetic modifications according to N-end rule and analyzed the mechanism for its difficult expression. We found that mutation of the second N-terminal amino-acid of the protein from lysine to alanine or truncating the N-terminal 16 amino-acid sequence resulted in successful expression of P14K. Moreover, fusion of a pelB leader and strep tag together (pelB-strep-P14K) at the N-terminus increased P14K expression. In addition, the pelB-strep-P14K was more stable than the strep-P14K.

Conclusions: Our results are not only useful for clarification of the role of P14K involved in the NHase maturation, but also helpful for heterologous expression of other difficult expression proteins.

Keywords: NHase, N-end rule, Pseudomonas putida, P14K, Stability

Background

Nitrile hydratase (NHase, EC 4.2.1.84) is composed of α- and β-subunits. The enzyme contains either a non-heme iron (Fe-NHase) [1] or non-corrin cobalt ion (Co-NHase) [2] in the active center and catalyzes the hydration of a nitrile to the corresponding amide, which is followed by several consecutive reactions: amide → acid → acyl-CoA, as catalyzed by amidase [3] and acyl-CoA synthetase [1], respectively. The metal ions in both Co-NHase and Fe-NHase are located in their α-subunits, which share a characteristic metal-binding motif [CXLC(SO2H)SC(SOH)] containing two modified cysteine residues: cysteine-sulfinic acid (αCys-SO2H) and cysteine-sulfenic acid (αCys-SOH) [1,4,5]. The apoenzyme is likely to be unmodified, according to previous studies on NHase [6] and a related enzyme, thiocyanate hydrolase (SCNase) [7]. The trafficking of metal ions into NHases is mediated by various “activator proteins” [8]. Fe-NHases require activators for functional expression in Rhodococcus sp. N-771 [9], Pseudomonas chlororaphis B23 [10] and Rhodococcus sp. N-774 [11]. A proposed metal-binding motif, CXCC, in the NHase activator of Rhodococcus sp. N-771 has been identified and the activators for Fe-type NHases have been shown to act as metallochaperones [12]. For the two Co-NHases (L-NHase and H-NHase) in Rhodococcus rhodochrous J1, cobalt incorporation has been found to be dependent on self-subunit swapping: the activator protein exists as a complex with the α-subunit of NHase, the cobalt incorporation involves the swapping of the cobalt-free α-subunit of the cobalt-free NHase with the cobalt-containing α-subunit of the complex [13-15]. NHase in Pseudomonas putida NRRL-18668 and acetonitrile hydratase (ANHase, an NHase
that catalyzes the hydration of small aliphatic nitriles) from *Rhodococcus jostii* RHA1 are also Co-NHases, in which P14K and AnhE, respectively, are essential for NHase maturation [16,17]. However, their gene organizations are quite different from those of L-NHase and H-NHase. The structural genes of L-NHase and H-NHase have the order <β-subunit > <α-subunit > <self-subunit swapping chaperone>, while those in ANHase and the NHase of *P. putida* NRRL-18668 have the order <α-subunit > <AnhE > <β-subunit> [16] and <α-subunit > <β-subunit > <P14K> [17], respectively, with the latter protein being identical to the metallochaperone in Fe-NHase except that the molecular mass of the protein in Fe-NHase is larger than P14K. While AnhE has been found to act as a metallochaperone (not as a self-subunit swapping chaperone) during cobalt incorporation into ANHase [16], very recently, we discovered that cobalt incorporation into the NHase of *P. putida* NRRL-18668 is also dependent on the self-subunit swapping, and the P14K is a complex with the α-subunit [18]. However, the P14K is difficult to be heterogeneously expressed, though a strep tag containing P14K was expressed, its low expression level and low stability retard the further clarification of its detailed role for cobalt incorporation.

Heterologous expression systems are commonly used for protein research. Protein degradation in heterologous expression systems often leads to failure for the isolation of proteins of interest. Intracellular protein degradation plays an essential role in many physiological processes by removing damaged polypeptides and proteins that harbor specific destruction tags. N-end pathway degradation relates the metabolic stability of a protein to the N-terminal residue of that protein [19]. The N-end rule defines the stability of proteins according to the nature of their N-terminal residues. These residues are classified as stabilizing and destabilizing residues, which serve as recognition determinants for protein degradation [19-21]. In bacteria such as *E. coli*, the N-end rule pathway is present. According to the N-end rule, amino-terminal arginine, lysine, leucine, phenylalanine, tyrosine and tryptophan confer 2 minute half-lives to proteins, while the other lysine, leucine, phenylalanine, tyrosine and tryptophan residues have the order < [24], reducing the distance between the promoter region and target gene [25] and gene codon optimization [26]. To enhance the expression of P14K, the putative RBS (CTGGAG) within the B gene (encoding the β subunit) (Figure 2A) was replaced with an enhanced RBS (AAGGAG) between gene B and gene P (encoding P14K) during the construction of the plasmid pET-ABP (Figure 3). Simultaneously, the plasmid pET-PAB, with the gene order <P > <A > <B>, was constructed. In this plasmid, the distance between the promoter sequence and the P14K gene was shortened and the strong RBS was inserted upstream of each gene (Figure 3).
Moreover, plasmid pET-ABPo, with optimized P14K gene codons and a strong RBS for each gene, was also constructed (Figure 2B). However, P14K was not abundantly expressed by the transformants harboring pET-ABP, pET-PAB or pET-ABPo (Figure 4A).

**Successful heterologous expression of P14K**

The N-end rule states that the half-life of a protein is determined by the nature of its N-terminal residue [19-21]. This fundamental principle of proteolytic regulation is conserved from bacteria to mammals [19]. N-terminal arginine, lysine, leucine, phenylalanine, tyrosine and tryptophan confer 2 minute half-lives to proteins, while the other N-terminal residues confer greater than 10 hour half-lives to the same proteins [22,23] (Figure 1). We analyzed the N-terminal amino-acids of some weakly expressed NHase activators from *Bordetella petrii* DSM 12804, *P. putida* NRRL-18668, *Bacillus* BR449 and *Bacillus* RAPc8. Surprisingly, we found that all of the second N-terminal amino-acids of the NHase activators in these four strains are lysine (K) (Table 1). Thus, it may be that N-end rule degradation leads to difficulty with the expression and isolation of P14K. Based on this speculation, we designed a mutant gene, ABP(K2A), in which the Lys-2 in the N-terminus of P14K was substituted with Ala in the plasmid pET-ABP(K2A). In addition, we analyzed the secondary structure of the P14K as predicted by JPRED3 (http://www.compbio.dundee.ac.uk/www-jpred/index.html) and found the N-terminal 16 amino-acid group is in an N-terminal loop (Figure 4B) and the 17th amino-acid is a Gly that could avoid P14K degradation effectively according the N-end rule [22,23]. Therefore, we constructed plasmid pET-AB(△N-P), in which the N-terminal...
16 residues of P14K were deleted, the second N-terminal amino-acid in the truncated P14K was Gly. The transformants harboring pET-ABP(K2A) and pET-AB(△N-P) were used for NHase and P14K expression. As shown in Figure 4C, each mutant P14K was successfully expressed and the crude NHase activity in the cell-free extracts of each transformant was similar to that of the transformant harboring pET-ABP(120.5 U/mg). However, compared with the expression of P14K using the transformant harboring pET-AB(strep-P), the expression level of P14K in the two mutants was not improved (Figure 4C).

**High P14K expression yield**

It has been reported that exporting a protein to the periplasm to enhance its stability can be regarded as an effective strategy to optimize the production of recombinant proteins [27,28]. To yield large amounts of P14K in recombinant E. coli cells, we attempted to secrete P14K into the periplasmic space. As P14K formed a complex with the α-subunit of NHase [18], we designed a mutant gene (pelB-strep-P14K) in which the pelB signal peptide was added upstream of the A and strep-P genes in the plasmid pET22b-(pelB-A)pelB-strep-P). The transformant harboring pET22b-(pelB-A)pelB-strep-P) was used for recombinant P14K expression. Although no target protein was observed in the culture supernatant, a large amount of the full-length pelB-strep-P14K and pelB-α subunit (the pelB signal peptides were not cut off in either) were detected in the cell-free extract (Figure 5A). In addition, the enzyme activity in the cell-free extract was comparable to that of the wild-type NHase.

**Stability of pelB-strep-P14K**

To investigate the mechanism of how the fusion protein pelB-strep enhances the recombinant expression of P14K, we compared the difference in the protein stability between the purified P14K-containing activator complex [α-(streP-P14K)] and [α-(pelB-strep-P14K)]. SDS-PAGE analysis was carried to investigate the stability of cobalt-containing [α-(streP-P14K)] and [α-(pelB-strep-P14K)] (a culture containing the cobalt ion) during storage at room temperature. As shown in Figure 5B, the pelB-strep-P14K band from the

**Table 1 N-terminal amino-acid sequence of NHase activators in various strains**

| Strain               | N-terminal amino-acid sequence          |
|----------------------|-----------------------------------------|
| Bordetella petri DSM 12804 | M K DERLPLP (YP_001630019.1)          |
| Pseudomonas putida NRRL-18668 | M K DERFPLP (P14K in this study*)      |
| Bacillus BR449        | M K SCENQPN (AAE69003.1)               |
| Bacillus RAPc8        | M K SCENQPN (AAS84452.1)               |

Protein accession numbers are shown in brackets. * the correct N-terminal amino-acid sequence of P14K was identified by our previous work [18].
recombinant [α-(pelB-strep-P14K)] complex decayed to 90% of the original intensity after 2 days of storage and eventually to 60% after 6 days. However, the strep-P14K band from the [α-(strep-P14K)] complex decreased to 60% after 1 day and to 10% after 2 days. The finding that pelB-strep-P14K is far more stable than strep-P14K indicated that thermal stability may be a key factor in P14K expression.

Conclusions
In conclusion, the activator P14K from *P. putida* NRRL-18668 was successfully expressed based on the N-end rule degradation, the stability of the P14K was improved by adding a pelB signal peptide. Further study of the influence of P14K on the maturation of NHase is currently underway. The strategy used for P14K expression in this study may be useful for the heterologous expression of other difficult expression proteins.

Methods
Bacterial strain and plasmids
NHase and the P14K gene (*ABP*) were cloned from *P. putida* NRRL-18668. *E. coli* BL21 (DE3) was used as the host for the plasmid pET-24a(+), which was used for *ABP, ABP*, *PAB, ABP*, *ABP-* (strep-P), *ABP*(K2A), *ABP*-N-P) and A(strep-P) expression. The plasmid pET-22b was used for (pelB-A)B(pelB-strep-P) expression.

Construction of plasmids
The genomic DNA of *P. putida* NRRL-18668 was isolated for *ABP* cloning with the primers A-up and P-down (Table 2 and Figure 3). The PCR products were digested and ligated into pET-24a(+) to generate the plasmid pET-ABP. The recombinant plasmid pET-ABP

Table 2 Oligonucleotide primers used in this study

| Name          | Sequence                                                                 |
|---------------|--------------------------------------------------------------------------|
| A-up          | 5'-GAATTCATTATGGGCAATCACACACGC-3'                                      |
| P-down        | 5'-CCGGAATCTCTCAGCCATAGCGGAACCGA-3'                                     |
| B-down(rbs)   | 5'-ATATCTATATCTTTCACGGCTGTCAGCTATGTC-3'                                 |
| P-up(rbs)     | 5'-TGAAGAAGATATATGATAGGAAAGACG-3'                                      |
| P-up          | 5'-GAATTCATTATGAAAGACGGAACCTTT-3'                                      |
| P-down(PAB)   | 5'-CATATCTATATCTTTCAGCCATTGCAGGATGTC-3'                                 |
| A-up(PAB)     | 5'-TGAAGAAGATATATGATAGGAAAGACGGAACCGA-3'                                |
| B-down(PAB)   | 5'-CCGGAATCTCTCAGCTGTCAGCTATGTC-3'                                     |
| Po-up         | 5'-GAATTCATTATGAAAGACGGAACCTTT-3'                                      |
| Po-down       | 5'-CCGGAATCTCTCAGCCATAGCGGAACCGA-3'                                     |
| B-down(strep) | 5'-GCGGGGTGGCCATGACCGATCGATCTATATCTTCTTTCAGCCATATGTCAGCTATGTC-3'        |
| P-up(strep)   | 5'-GCGGGGTGGCCATGACCGATCGATCTATATCTTCTTTCAGCCATATGTCAGCTATGTC-3'        |
| K2A-up        | 5'-GAATTCATTAGGGCTATGTCATCTATATCTTCTTTCAGCCATATGTCAGCTATGTC-3'          |
| K2A-down      | 5'-GCAATGGAACCGTGTCGCATCTATATCTTCTTTCAGCCATATGTCAGCTATGTC-3'            |
| P-up(N)       | 5'-GAATTCATTATGAAAGATATATGATAGGAAAGACG-3'                               |
| A-down(strep) | 5'-GCGGGGTGGCCATGACCGATCGATCTATATCTTCTTTCAGCCATATGTCAGCTATGTC-3'        |
| secA-up       | 5'-GAATTCATTGCGGCAATACACACGCCAT-3'                                     |
| secB-down     | 5'-GAATTCATTGCGGCAATACACACGCCAT-3'                                     |
| secP-up       | 5'-GACGGGTGGCCATGACCGATCGATCTATATCTTCTTTCAGCCATATGTCAGCTATGTC-3'        |
| secP-down     | 5'-GACGGGTGGCCATGACCGATCGATCTATATCTTCTTTCAGCCATATGTCAGCTATGTC-3'        |

Italicized letters denote the NdeI, EcoRI and HindIII recognition sites, respectively.
Expression and purification of enzymes and enzyme assay

*E. coli* BL21 (DE3) transformants containing the recombinant plasmids were grown at 37°C in TB medium containing CoCl$_2$$\cdot$6H$_2$O (0.05 g/l) and kanamycin (50 μg/ml) until culture $A_{600}$ reached 0.8. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM. The cells were then incubated at 24°C for 16 h.

All purification steps were performed at 4°C. The procedures were conducted with an AKTA purifier (GE Healthcare UK Ltd.). Potassium phosphate buffer (KPb) (10 mM, pH 7.5) containing 0.5 mM dithiothreitol (DTT) was used in the purification steps. Both NHase and its activator complex were purified with a HiTrap HP column (GE Healthcare UK Ltd.). The target proteins were eluted off the column with gradient concentrations of imidazole from 0 mM to 500 mM (40 mM, 80 mM, 200 mM, 300 mM and 500 mM) in 10 mM KPb. The preliminarily separated proteins were further purified with a Hiliad 16/60 Superdex 200 pg column (GE Healthcare UK Ltd.). The process of separation and purification was monitored by SDS-PAGE analysis.

NHase activity was assayed in a reaction mixture comprising 10 mM KPb (pH 7.5), 20 mM 3-cyanopyridine as a substrate and 0.1 μg enzyme in a total volume of 500 μl. The reaction mixture was incubated at 20°C for 20 min and terminated by addition of 500 μl of acetone. The activity of NHase was determined by monitoring the formation of nicotinamide in the reaction mixture with high-pressure liquid chromatography (HPLC) as previously described [13]. One unit of NHase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of nicotinamide per min at 20°C.

Competing interests

The authors declare that there are no competing interests.

Authors’ contributions

ZMZ, WJC and MK designed this study. YL, YQF, YCY, YTC and YYX performed the experimental work. ZMZ, WJC, YL and MK wrote the manuscript. ZMZ and MK collaborated in the coordination of the research and helped to draft the manuscript. All authors read and approved the submission of the manuscript.

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