Expression and purification of the kinase domain of PINK1 in *Pichia pastoris*

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**Abstract**

PTEN-induced putative kinase 1 (PINK1) is a Ser/Thr kinase that specifically localizes on the mitochondrial membrane. It cooperates with Parkin to regulate mitochondrial quality control. Mutations in PINK1 protein which account for 8–15% of Parkinson’s disease (PD), are the second most common cause of early-onset Autosomal Recessive Parkinson’s disease (AR-PD). The lack of methods for PINK1 heterologous expression and purification has slowed progress in the AR-PD research field. To pave the way for direct structural study of this important protein, in this study, we developed an efficient expression system of recombinant PINK1 kinase domain (rPINK1) using *Pichia pastoris* (*P. pastoris*). Our results showed that rPINK1 is best expressed in *P. pastoris* at 25°C induction. Additionally, we determined that the optimal induction time was 72 h and the optimal induction methanol concentration was 1% for the expression of rPINK1 in *P. pastoris*. Subsequent purification by Ni affinity chromatography (Ni-NTA) and cation-exchange chromatography (Mono S) produced the protein with purity higher than 95%. The pure rPINK1 was active to phosphorylate ubiquitin in a substrate phosphorylation assay. Overall, these studies provide the first effective method for heterologous expression and purification of the rPINK1 with a high purity. These findings can help contribute to further researches on the interactions study and biochemical characterization of PINK1.

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1. Introduction

Parkinson’s disease (PD) is a chronic progressive neurodegenerative disease that leads to bradykinesia and resting tremor [1]. It is caused by selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) during the response to mitochondrial damage [2]. PINK1 (Fig. 1A) is a mitochondrial membrane localized kinase that plays critical roles in identifying and targeting damaged mitochondria for degradation. It cooperates with Parkin (downstream factor of PINK1) in a signaling pathway to regulate mitochondrial quality control [3,4]. This process involves changing the fission and fusion of mitochondria, regulating the transport of mitochondria, initiating the removal of mitochondrial proteins, and mediating the biogenesis and autophagy of mitochondria [2,5]. Both PINK1 and Parkin mutations are associated with the early-onset Autosomal Recessive PD (AR-PD) [6,7] as a result of disrupting mitochondrial dynamic equilibrium and mitochondrial function [2,8].

Parkin is the ligase in the ubiquitin-proteasome system for degradation. The enzymatic activity of Parkin is inactive via auto-inhibition under steady state conditions [9]. PINK1 activates Parkin enzymatic activity not only through phosphorylation of the ubiquitin-like domain of Parkin (Ser65) [9,10] but also through phosphorylation of ubiquitin serine 65 in an ATP-dependent manner [11–14]. And the Km value of PINK1 for ATP is 74.6 ± 13.2 μM [15]. Unlike phosphomimetic Parkin (S65D), phosphomimetic ubiquitin (S65D) did bypass the function of PINK1 in cell [16,17]. This indicates that ubiquitin acts allosterically as a key molecular substrate of PINK1 in the activation of Parkin [13].

Despite the physiologically important functions of PINK1 kinase activity, there are no reports of obtaining high purity PINK1 in *vitro*. The *P. pastoris* expression system is an eukaryotic expression system developed in recent decades [18]. It is an ideal expression system with the advantages of inexpensive media, post-translational modification (phosphorylation, glycosylation and

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formation of disulfide bonds), processing target proteins into native conformations, and secretion of the recombinant proteins into the fermentation broth [18,19]. Moreover, the yield of recombinant protein can be improved significantly by high density fermentation [20]. However, there was no report about the expression of PINK1 in \textit{P. pastoris}. Here, we reported a method for heterologous expression and purification of the kinase domain of PINK1 in \textit{P. pastoris}, which will be useful for future PINK1 research.

2. Materials and methods

2.1. Materials

The human PINK1 gene was supplied by AddGene. The pPICZ\textsubscript{a}A plasmid (Invitrogen) and \textit{E. coli} JM109 (stock in our lab) were used for vector construction. \textit{P. pastoris} X-33 strain (Invitrogen) was used for expression. Restriction enzymes and T4 DNA ligase were purified by Takara. DNA purification kit and plasmid extraction kit were purchased from Axygen. Primers were synthesized by GeneScript. Nickel beads were purchased from GE Health Company. Penta-His antibody was purchased from Qiagen. The human PINK1 gene was supplied by AddGene. The pPICZ\textsubscript{a}A plasmid (Invitrogen) and \textit{E. coli} JM109 (stock in our lab) were used for vector construction. \textit{P. pastoris} X-33 strain (Invitrogen) was used for expression. Restriction enzymes and T4 DNA ligase were purchased from Takara. DNA purification kit and plasmid extraction kit were purchased from Axygen. Primers were synthesized by GeneScript. Nickel beads were purchased from GE Health Company. Penta-His antibody was purchased from Qiagen.

2.2. Construction of rPINK1 transformant

The kinase domain boundary of PINK1 (PINK1\textsubscript{148–515}) was determined as previously described [21]. And the theoretical isoelectric point and molecular weight of the recombinant kinase domain of PINK1 (rPINK1) were calculated online (http://web.expasy.org/protparam/). The DNA fragment encoding the kinase domain of PINK1 (rPINK1) were calculated online (http://web.expasy.org/protparam/). The DNA fragment encoding the kinase domain of PINK1 (rPINK1) were calculated online (http://web.expasy.org/protparam/). The DNA fragment encoding the kinase domain of PINK1 (rPINK1) were calculated online (http://web.expasy.org/protparam/). The DNA fragment encoding the kinase domain of PINK1 (rPINK1) were calculated online (http://web.expasy.org/protparam/).

2.3. Optimization of the fermentation conditions of rPINK1

Penta-His antibody was purchased from Qiagen. The human PINK1 gene was supplied by AddGene. The pPICZ\textsubscript{a}A plasmid (Invitrogen) and \textit{E. coli} JM109 (stock in our lab) were used for vector construction. \textit{P. pastoris} X-33 strain (Invitrogen) was used for expression. Restriction enzymes and T4 DNA ligase were purchased from Takara. DNA purification kit and plasmid extraction kit were purchased from Axygen. Primers were synthesized by GeneScript. Nickel beads were purchased from GE Health Company. Penta-His antibody was purchased from Qiagen.

2.4. Western Blotting

The fermentation supernatants were collected and resuspended in 10 ml BMMY medium to an OD\textsubscript{600} of approximately 5.0. 2 ml of the fermentation supernatants was incubated with nickel beads at 4 °C for 90 min. Then the mixture was loaded into a gravity flow column. The nickel beads were extensively washed with 5 column volumes of buffer A. rPINK1 eluted by buffer A with 250 mM imidazole. Eluted proteins were concentrated using a Millipore centrifugal filter (3 kDa), and then diluted 5 times with buffer B (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM DTT) by concentration and dilution. Purified protein was flash-frozen in liquid nitrogen and stored at −80 °C for activity assay. The concentration of rPINK1 was measured at the absorption of 280 nm by a DeNovix spectrophotometer after entered with the molecular weight and extinction coefficient. The molecular weight and extinction coefficient were calculated online (http://web.expasy.org/protparam/). The purity of rPINK1 was evaluated by SDS-PAGE and quantified by Quantity One software (Bio-Rad).

2.5. Protein purification of rPINK1

The rPINK1 transformant was fermented in 400 ml of medium under an optimum fermentation conditions (72 h, 25 °C and 1% methanol). 400 ml fermentation supernatant was concentrated into about 10 ml using a Millipore centrifugal filter (3 kDa), and then diluted 5 times with buffer A (50 mM Tris-HCl pH 8.0, 20 mM imidazole, 500 mM NaCl). The diluted protein sample was incubated with nickel beads at 4 °C for 90 min. Then the mixture was loaded into a gravity flow column. The nickel beads were extensively washed with 5 column volumes of buffer A. rPINK1 eluted by buffer A with 250 mM imidazole. Eluted proteins were concentrated using a Millipore centrifugal filter (3 kDa), and then diluted 5 times with buffer B (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM DTT) by concentration and dilution. Purified protein was flash-frozen in liquid nitrogen and stored at −80 °C for activity assay. The concentration of rPINK1 was measured at the absorption of 280 nm by a DeNovix spectrophotometer after entered with the molecular weight and extinction coefficient. The molecular weight and extinction coefficient were calculated online (http://web.expasy.org/protparam/). The purity of rPINK1 was evaluated by SDS-PAGE and quantified by Quantity One software (Bio-Rad).

2.6. Substrate phosphorylation assay in vitro

To evaluate the property of our purified rPINK1, the kinetic study of rPINK1 phosphorylation assay was performed as previously described [13]. His-tagged ubiquitin was cloned, expressed, and purified as previously mentioned [13]. Reaction of 30 μg rPINK1 and 2.4 μg ubiquitin in the kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl\textsubscript{2}, 10 mM DTT, 1 mM ATP) was incubated at 30 °C for 1, 5, 15, 30, 45 and 60 min, respectively. The reaction was stopped by adding 2 × SDS sample buffer and boiling. Phosphorylated and unphosphorylated proteins were separated by 50 μM Phos-Tag SDS-PAGE (WAKO). Then the gel was soaked in the transfer buffer with 1 mM EDTA for 20 min to remove the Mn\textsuperscript{2+} before being transferred to PVDF membrane. His-tagged proteins were detected by Western Blotting using Penta-His antibody. The slower migrated protein was the phosphorylated protein.

3. Results

3.1. Construction of rPINK1 transformant

PINK1(148–515) was cloned into pPICZ\textsubscript{a}A with a C-terminal His-tag (Fig. 1B). The constructed pPICPINK1 was verified by Xhol/XbaI digestion (Fig. 1C) and sequencing. We named the recombinant protein PINK1(148–515) as rPINK1. The theoretical isoelectric point and molecular weight of rPINK1 was 7.34 and 40.53 kDa, respectively.
The recombinant plasmid pPICPINK1 and empty vector pPICZαA were linearized by the restriction enzyme BglII and transformed into P. pastoris X-33. The expression of target protein in fermentation supernatant was evaluated by Western Blotting using an anti-His tag antibody. The results showed that rPINK1 was expressed but partly degraded at 30°C while no target protein expression in empty pPICZαA transformant (Fig. 1D). The pPICPINK1 transformant (Fig. 1D, line 4) with a higher expression level was further verified by PCR (Fig. 1E). Then we selected the positive transformant (Fig. 1D, line 4) for further optimization and purification.

### 3.2. Optimization of rPINK1 expression

To find out the best expression condition for rPINK1, we optimized the induction time, temperature, and concentration of methanol. We checked the protein expression at different induction times from 24 h to 120 h. The results showed that rPINK1 was expressed but partly degraded at 30°C while no target protein expression in empty pPICZαA transformant (Fig. 1D). The pPICPINK1 transformant (Fig. 1D, line 4) with a higher expression level was further verified by PCR (Fig. 1E). Then we selected the positive transformant (Fig. 1D, line 4) for further optimization and purification.

The results showed that rPINK1 was expressed well when the temperature was higher than 21°C (Fig. 2C). Degradation product was detected when the temperature was higher than 25°C (Fig. 2C). We chose 25°C as the best temperature for expression. Various concentrations of methanol were tested at 25°C induction. The results showed that the expression level of rPINK1 was the highest at a concentration of 1% (Fig. 2D).

### 3.3. Purification of rPINK1

We induced the expression of rPINK1 at the best condition (72 h, 25°C and 1% methanol). Fermentation supernatant was collected and concentrated 10 times for SDS-PAGE analysis. The results showed that rPINK1 was well expressed and very soluble in the supernatant (Fig. 3A). The concentrated supernatant of rPINK1 was subsequently purified by His-tag affinity purification. The purity of rPINK1 can reach 90% after His-tag affinity purification (Fig. 3A). Based on the theoretical isoelectric point of rPINK1, we chose a strong cation exchange Mono S to further purify it at pH 6.0. rPINK1 was eluted from Mono S at about 300 mM NaCl (Fig. 3B). It was almost 95% pure after Mono S chromatography (Fig. 3C) and able to be concentrated to 6 mg/ml. Then we analyzed the homogeneity of rPINK1 by Size-exclusion chromatography (Superdex 200). Only a single peak was observed (Fig. S1). It indicating rPINK1 is a homogeneous monomer. In summary, based on the optical...
density of the SDS-PAGE gel and the total protein level, the overall yield of the rPINK1 was about 10 mg/l. And the yield of purified rPINK1 was about 1.5 mg/l of culture. The total recovery rate of rPINK1 was about 15%.

3.4. Phosphorylation of ubiquitin by rPINK1

To perform a time course assay of rPINK1 phosphorylating ubiquitin, the purified His-tagged ubiquitin (Fig. 4A) and rPINK1 were added into the reaction system. Then we used Phos-Tag SDS-PAGE and Western Blotting to detect and differentiate phosphorylated protein from un-phosphorylated protein. The phosphate group of phosphorylated protein has specific affinity with acrylamide-pendant Phos-tag ligand. Thus, the phosphorylated protein will immigrate slower in Phos-Tag SDS-PAGE. The result showed that ubiquitin was phosphorylated by rPINK1 in a time dependent manner (Fig. 4B, bottom panel). In addition, we also observed some auto-phosphorylated rPINK1 bands (Fig. 4B, upper panel), which indicating that our purified rPINK1 has multiple auto-phosphorylation sites. This result was consistent with the previous studies that PINK1 had several auto-phosphorylation sites [24,25]. Moreover, we calculated the Km value of rPINK1 for ATP in an auto-phosphorylation assay as previously described [15]. The Km value of rPINK1 for ATP was 90.3 ± 6.4 μM, which was close to previous 74.6 ± 13.2 μM.

4. Discussion

PINK1 is an important protein related to AR-PD. It is constitutionally localized to healthy mitochondria, and rapidly cleaved and degraded by proteasomes. However, it accumulates on the outer mitochondrial membrane in damaged mitochondria. PINK1
recruits Parkin to these unhealthy mitochondria and initiates the Parkin mediated mitochondrial autophagy [26,27] to regulate the mitochondrial fidelity [2,28]. It indicates that PINK1 plays a role as a sensor protein for mitochondrial damage signaling during the generation of AR-PD [29].

As previous studies reported, many attempts have been made to express PINK1 using an E. coli expression system [30,31]. However, most of the recombinant PINK1 expressed in E. coli is poorly soluble and extremely unstable. Additionally, some researchers expressed PINK1 using an insect cell expression system [21]. However, the recombinant PINK1 was also degraded to a size of about 30 kDa. E. coli [30,31] or insect cell expression systems [15,21] both express recombinant PINK1 intracellularly. Because PINK1 could be cleaved by the mitochondrial processing peptidase (MPP) and PARL under steady state conditions [32], PINK1 might be cleaved by the endogenous proteases in E. coli and insect cell.

In this study, we used secretory expression system P. pastoris to avoid intracellular degradation issues. Additionally, P. pastoris is a eukaryotic cell with powerful posttranslational capabilities. The recombinant protein can be secreted into the endogenous proteases in steady state conditions [32], PINK1 might be cleaved by the endogenous proteases in E. coli and insect cell.

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In summary, we report the first method of effectively expressing and purifying the kinase domain of PINK1 from P. pastoris. We optimized the induction time, temperature and methanol concentration for rPINK1 expression. After two-step purification, the purity of rPINK1 is higher than 95%. Additionally, the activity of rPINK1 was verified by ubiquitin phosphorylation assay. In conclusion, this paper provides high purity protein materials for biochemical studies and the discovery of the interacting proteins of PINK1 and other biochemical studies.

**Competing interests**

The authors declare that they have no competing interests.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2016.08.010.

**References**


