Expression of recombinant mouse spastin in *E. coli*

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Abstract. Mutations of the gene *SPAST* that encodes a microtubule severing enzyme, spastin, are the most frequent cause of Hereditary spastic paraplegia (HSP) disease. HSP is heterogeneous group of inherited neurodegenerative disorders characterized predominantly by progressive lower limb spasticity and weakness. Spastin belongs ATPase associated with various cellular activities (AAA) protein family and catalyzes microtubule severing. Spastin in mouse and human are highly identical in protein sequence and several spastin mutation models in mice have been generated in order to evaluate the significance of spastin loss-of-function in mammals. Expression and purification of spastin and the mutant variants determined in patients will facilitate the structure-function relationship study of spastin. Here I systemically optimized the expression condition of a truncated version of mouse spastin in *E. coli*. The recombinant protein and a mutant were further purified for ATPase activity assay.

1 Introduction

In human, mutations in the *SPAST* (previously known as *SPG4*) gene that encodes spastin are the most common cause of Hereditary Spastic Paraplegia (HSP). Autosomal-dominant HSP-*SPG4* account for 15–40% of all HSP cases depending upon the ethnic background of patients. HSP is a group of neurodegenerative diseases with significant clinical and genetic heterogeneity, characterized by predominantly, but not exclusively, slow progressive weakness of the lower limbs and spastic paraplegia^[1].

Human *SPAST* gene locus on 2p22.3 contains 17 exons and encodes a microtubule severing enzyme, spastin of 616 amino acids. Spastin is a member of ATPase associated with various cellular activities (AAA) protein family. AAA domain between residues 342 to 599 is located on the C-terminus of spastin protein and is involved catalyzing microtubule cleavage^[2]. Spastin also contains microtubule-binding domain (MTBD), hydrophobic domain (HD) that forms the hairpin structure, and the domain of microtubule interacting and trafficking (MIT) domain. MTBD, spanning residues 270 to 328, promotes the binding of spastin and microtubules before severing microtubules^[2]. The crystal structure of the spastin from *Drosophila* showed that spastin hexamer forms a ring with a prominent central pore and six radiating arms that may dock onto the microtubule. Spastin performs the microtubule severing activity depending on the disordered and negatively charged C-terminal tails of the tubulin. Study has revealed that spastin use its pore loops to tug the C-terminal tail of tubulin and generates a mechanical force accompanied with hydrolysis of ATP^[1,4].

The pathogenesis of HSP linked to spastin mutation

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has not been fully determined yet. In order to evaluate the significance of spastin loss-of-function in mammals. Mice harboring deletion of *Spast* exons 5 to 7 show axonal swellings in both descending and ascending tracts of the spinal cord from 4 months of age^[5]. A convenient method to obtain purified spastin and its mutants identified in patients will facilitate studies on its structure-function relationship.

2 Methods

2.1 Plasmid construction and mutagenesis

The longest open reading frame (1845 bp) of mouse spastin was synthesized (General biosystems, China). The truncated spastin (spastin-C389) lacks the N-terminal 225 amino acids. Truncated-spastin was constructed to plasmid with His-GST tag using ligation independent cloning method. Constructed expression plasmid was confirmed by DNA sequencing (Tsingke Biotech, China). The plasmid was amplified with two mutagenic primers and Pfu polymerase (Tiangen, China), which introduced a E439A mutation in the AAA domain. Template DNA was removed by digesting with DpnI (New England Biolabs, Inc.) for 1 h at 37°C; the DNA was then transformed into *E. coli* cells and single clones were isolated. The presence of the mutation was verified by DNA sequencing. Using this method, I generated a previously described inactive versions for spastin^[6], sequenced by the company (Tsingke Biotech).

2.2 Expression of recombinant spastin in *E. coli*

Recombinant protein His-GST-Spastin-C389 was grown

in Rosetta (DE3) at 37°C cells until the OD600 reached around 0.8. Then spastin-C389 expression was induced with different concentrations of IPTG, then incubated at different temperature, at 220 rpm. The induction conditions (37°C for 4 h, 30°C for 6 h, 16°C for 16h with 0.4 mM IPTG as well as 30°C for 6 h with 0.5mM IPTG) were tested to optimize to get the most soluble recombinant spastin-C389. After induction, the cells were harvested by centrifugation and the pellet was resuspended in 2 mL lysis buffer (20 mM HEPES, pH 7.4, 250 mM KCl, 25 µg/mL DNase, 25 µg/mL Lysozyme, 10 mM PMSF, 10 mM β-ME), followed by sonication. The supernatant and pellet were collected by centrifugation and pellet was resuspended with 2 mL buffer (20 mM HEPES, pH 7.4, 250 mM KCl). The expression of proteins in supernatant and pellet were solved by 8% SDS-PAGE and visualized by staining with Coomassie brilliant blue (CBB).

2.3 Purification of recombinant proteins

Plasmid pET-His-GST-spastin-C389 and mutation pET-His-GST-spastin-C389-E439A were expressed in Rosetta (DE3) cells with induction at 30°C for 6 h with 0.5 mM IPTG. For purification, I adapted from published paper^[7]. Bacteria cells were resuspended in lysis buffer (20 mM Tris/HCl, pH 7.5, 300 mM NaCl, 2 mM MgCl₂, 1 mM ATP, and protease inhibitors) and sonicated. After centrifugation, the supernatant was collected and incubated with glutathione Sepharose (GenScript, China) for 1 h at 4°C with shaking. The resin was washed with 10-fold beads bed volume of wash buffer (20 mM Tris/HCl, pH 7.5, and 500 mM NaCl). After washing, fusion proteins were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM MgCl₂, 5 mM DTT, and 20 mM reduced glutathione). Elution fractions were collected in several tubes with each fraction of 0.5 mL. Concentrations of all collected fractions were measured using Nanodrop and the proteins were checked by SDS-PAGE. Fractions containing target protein band were combined and dialyzed against 2 L HEPES dialysis buffer (20 mM Hepes/NaOH, pH 7.5, 100 mM NaCl, 3 mM MgCl₂, and 5 mM DTT) overnight at 4°C. Dialyzed protein was concentrated and concentration was measured with BCA kit according the manufacture's protocol.

2.4 ATPase assay using malachite green

ATPase activity was measured using the malachite green colorimetric assay^[7,8]. A series of concentrations of sodium phosphate were used to generate a standard curve. Enzyme reactions were performed with 0.2 mM ATP as substrate supplemented with 1.4 µg enzyme in a 100 µL reaction system. Inactive spastin mutant and denatured enzymes groups were also performed as control. Reactions were incubated at 37°C for 3 h. After the reaction, 0.4 mL MG-AM color reagent was added and mixture was vortex immediately to terminate the reaction. After 2 min, 50 µL of 34% citrate solution was added to the mixture with immediate vortex. After 5 min, the

absorbance (OD 650nm) was recorded. Finally, the amount of phosphate generated of each reaction group were calculated according to the standard curve. Each reaction group was repeated for triplicate.

3. Results

3.1 Optimization of conditions for spastin-C389 expression in *E. coli*

Firstly, plasmid to express truncated version of mouse spastin (spastin-C389) in bacteria was constructed. Spastin-C389 contains all domains requirement for severing activity. In order to get the large amount of soluble recombinant spastin, we optimized the expression condition of spastin-C389 in *Escherichia coli* (*E. coli*) cell, Rosetta (DE3). The induction temperature was tested at 37°C, 30°C, 16°C and the IPTG induction concentration was tried as 0.4 mM and 0.5 mM respectively (Fig. 1). Based on the sequence of constructed plasmid, the molecular weight of recombinant protein was around 72 kDa. By analysis of the protein induced at 37°C and 30°C with 0.4 mM IPTG, the target protein in supernatant induced at 30°C was more than that induced at 37°C (Fig. 1A). We then also tried to induce the protein expression at 16°C with 0.4 mM IPTG. But target protein was expressed much less in the supernatant (Fig. 1). Also, it seemed that protein in supernatant after addition of IPTG was not expressed more than that without addition of IPTG. It may be due to the leaky expression of protein. So, we added glucose to suppress the level of uninduced protein expression in Rosetta cells. However, addition of glucose didn't make soluble recombinant protein express more (Fig. 1B). Finally, I tried to induce the protein expression at 30°C with 0.5 mM IPTG (Fig. 1C). Compared to other condition, the induction condition that 30°C with 0.5 mM IPTG yielded the most amount soluble recombinant protein.

Fig. 1. Protein expression of spastin-C389 in Rosetta (DE3) cells by different induction conditions. The supernatant and precipitation of cell lysate were fractionated by centrifugation and proteins were separated by 8% SDS-PAGE stained by CBB. (A) Protein was induced at 37°C for 4 hours with 0.4 mM IPTG, and at 30°C with 0.4 mM IPTG for 6 hours. It showed that soluble spastin at 30°C was more than 37°C. (B) Protein was induced at 16°C for 16 hours with 0.4 mM IPTG as well as at 30°C for 6 hours with 0.4 mM IPTG, supplementation of 0.3% glucose. Soluble recombinant protein level was not improved in both conditions. (C) Recombinant protein induced at the condition of 30°C with 0.5 mM IPTG for 6 hours was found to have more soluble expression compared to other

conditions. M.: marker; pet.: pellet; sup.: supernatant; -: without IPTG; +: addition of IPTG

3.2 Purification of recombinant spastin-C389 and its E439A mutant

The plasmids pET-His-GST-spastin-C389 and mutation pET-His-GST-spastin-C389-E439A were transformed to Rosetta (DE3) and protein expression was induced at the optimized conditions. Then recombinant proteins were purified by Glutathione-Sepharose. Expressed soluble proteins and purified target proteins were detected by 8% SDS-PAGE stained with Coomassie brilliant blue and confirmed by immunoblotting with anti-GST antibody (Fig. 2).

Fig. 2. SDS-PAGE analysis of purified His-GST-tagged recombinant Spastin-C389 and Spastin-C389-E439A by Glutathione-Sepharose resin. (A) Crude expressed cells after sonication are indicated as supernatant. Flow through after binding with beads, wash fractions and elution fractions passed through Glutathione-Sepharose were collected and separated by SDS-PAGE, and visualized after staining with CBB. (B) The final dialyzed and concentrated His-GST-Spastin-C389 protein was separated by SDS-PAGE and visualized by staining with CBB. Recombinant protein was also identified by immunoblotting with anti-GST antibody. (C) Similar to spastin-C389 protein, different fractions during purification of mutated protein His-GST-Spastin-C389-E439A were separated and visualized after staining with CBB. The molecular size of target protein band is 72 kDa. M.: marker.

3.3 Verification of the recombinant spastin activity

Spastin belongs to the AAA ATPase family. The conserved amino acids in the AAA motifs contribute to ATP binding and hydrolysis. In this study, the purified Spastin-C389 containing AAA domain was expected to have ATPase activity^[7]. The Spastin-C389-E439A introduced mutation containing the mutation in the AAA domain, which deactivate the enzyme^[7], was used as a control. Thus, the ATPase activity of recombinant Spastin-C389 and its mutation purified from *E. coli* were determined by malachite green colorimetric method. This assay based upon the detection of inorganic phosphate ions (Pi), generated by the enzymatic hydrolysis of ATP. The determination of Pi was achieved through the

formation of a phosphomolybdate complex and subsequent reaction with malachite green (MG) reagent. Here, denatured enzymes were also employed as a negative control. The recombinant Spastin-C389 showed significant ATPase activity, whereas E439A mutant has no detectable activity (**Figure 3**). These results suggested the purified protein remained enzymatically active.

Figure 3. ATPase activity analysis of purified recombinant spastin by malachite green colorimetric method. WT (His-GST-Spastin-C389) protein showed significant activity comparing to the control mutated protein: EA (His-GST-Spastin -C389-E439A). Also, denatured WT enzyme and mutation enzyme were used as negative control. For each group, assay was performed by triplicates. The data was represented with mean $\pm$ SEM and analyzed with Student's t-test. Significance level was set at 95%. *** <0.001

4. Conclusion

It has been reported GST tagged mouse spastin-C389 showed microtubule severing activity^[7]. In this study, we used His-GST-tagged spastin-C389 and optimized the protein expression in *E. coli* cells to yield soluble protein. Also the purified truncated-spastin was examined to show ATPase activity with a mutation as a control, which has been reported to be inactive^[7], suggesting that His-GST tag did not affect the truncated-spastin activity. Using this purified protein, it can be performed spastin severing activity assays.

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