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Expression and purification of a difficult sarcomeric protein: telethonin

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Abstract

Telethonin anchors the N-terminal region of titin in the Z-disk of the sarcomere by binding to two immunoglobulin-like (Ig) domains (Z1 and Z2) of titin (Z1Z2). Thereby telethonin plays an important role in myofibril assembly and in development and functional regulation. The expression and purification of recombinant telethonin is very challenging. In previous studies, recombinant telethonin expressed from *E. coli* was refolded in the presence of Z1Z2. Here, we report various strategies to establish a reliable and efficient protocol for the preparation of telethonin and titin Z1Z2 protein. First, a co-expression strategy was designed to obtain soluble telethonin/Z1Z2 complexes. The concentration of antibiotics and the type of expression vector were found important to achieve high yields of purified complex. Second, the five cysteine residues of telethonin were mutated to serine to avoid severe problems with cysteine oxidation. Third, a short version of of telethonin (telethonin$_{1\text{-}90}$) was designed to avoid proteolytic degradation observed for longer constructs of the protein. The short telethonin protein was forms a highly stable complex with Z1Z2 with no degradation being observed for 30 days at 4°C. Fourth, an improved refolding protocol was developed to achieve high yields of Z1Z2/telethonin complex. Finally, based on the observation that Z1Z2 and telethonin$_{1\text{-}90}$ assemble into a 2:1 complex, a single chain fusion protein was designed, comprising two Z1Z2 modules that are connected by flexible linkers N- and C-terminally of the telethonin$_{1\text{-}90}$. Expression of this fusion protein, named ZTZ, affords high yields of soluble expressed and purified protein.

Keywords: telethonin; Z1Z2; protein expression; protein purification; co-expression

1. Introduction

The muscle Z-disk defines the border between adjacent sarcomere units in the myocyte cytoskeleton. In the Z-disk at least three sarcomere filament systems are anchored and aligned, comprising titin, actin and nebulin [1]. The giant muscle protein titin is the largest protein known with a molecular weight of up to 4 MDa [2, 3], spanning one half of the sarcomere in striated and cardiac muscle [4]. In the Z-disk, two titin molecules are assembled into a complex involving interactions of the two N-terminal immunoglobulin-like (Ig) domains Z1 and Z2 (Z1Z2).The small 167-residue protein residue protein telethonin was found to mediate this assembly REF. Telethonin is the most abundant transcripts in skeletal muscle [6, 7]. In the Z-disk, many proteins have been found to interact strongly with telethonin, such as the muscle LIM protein (MLP), the potassium channel regulation protein Mink, the FATZ protein and the muscle growth factor myostatin [4]. Mutations in the telethonin genes are associated with limb-girdle musclar dystrophy type 2G (LGMD 2G), as well as hypertrophic and dilated cardiomyopathy [8]. These findings demonstrate that telethonin plays an important role in myofibril assembly and in development and functional regulation [9].

The Z1Z2/telethonin complex has been suggested to be critical for the Z-disk structure and for anchoring titin in the Z-disk [10]. Biochemical studies showed that the Z1Z2/telethonin complex is required for the sarcomere integrity and muscle growth [11]. Biochemical data and the crystal structure of the Z1Z2/telethonin complex reported by Zou et al. REF revealed that the titin Z1Z2 protein is assembled into an antiparallel (2:1) sandwich complex with telethonin [5]. The structural integrity of the Z-disk depends upon the tight network of hydrogen bonds between Z1Z2 and telethonin. Consequently, the complex can resist extremely high mechanical loads. Molecular dynamics simulations and single-molecule force spectroscopy experiments demonstrated that the Z1Z2/telethonin complex resists large mechanical force (about 700 pN) by β strand crosslinking, in contrast to relatively low mechanical force (about 168 pN) for Z1Z2 alone. The interaction between Z1Z2 and telethonin represents the strongest protein-protein interaction observed so far [12, 13]. In this respect, the complex is perfect for firmly anchoring the giant muscle protein titin in the Z-disk [4, 14].

Previous studies indicated that telethonin in the unbound state may exist in a distinct conformation compared to when bound to Z1Z2 and that the isolated telethonin is not stable [4]. These results are consistent with the observation that purified telethonin tends to aggregate in solution, and that telethonin is usually expressed into inclusion bodies [9]. It is thus believed that Z1Z2 plays a role in stabilizing the fold of telethonin [4]. The Z1Z2/telethonin complex cannot be formed by simply mixing the two components. Z1Z2 preferentially adopts semi-extended conformations in solution, with close-hinge arrangements [15], while in the complex with telethonin Z1Z2 adopts a fully extended conformation [9]. This conformation probably results from an induced fit of Z1Z2 upon binding to its partner. The predicted secondary structure of free telethonin is completely different than the secondary structure observed in the complex. This indicates that complex formation involves a mutually induced conformational change [4, 15].

Therapeutic peptides and proteins have attracted considerable interest as they exhibit

high binding affinity, high specificity, high solubility and low toxicity. However, the short half-life of many peptides and proteins, typically from a few minutes to a few hours resulting from the enzymatic degradation and renal clearance, hampers the application of these peptides and proteins in clinical treatment [16]. In order to prolong the half-life of these peptides and proteins, various strategies have been proposed. Conjugating poly-ethylene glycol (PEG) regions to the peptides and proteins by chemical methods was proved to be an effective way to extend the half-life of an attached peptide or protein by steric hindrance [17]. Genetically encoded fusion of a peptide or protein to a larger protein to increase the size is also widely used to prolong the retention time in circulation. Different fusion tags have been proposed, such as proteins with naturally long half-lives, such as human serum albumin or the Fc domain of IgG, or non-natural polypeptide stretches, such as XTEN [18], PAS [19], and HAP [20]. The molecular weight of the Z1Z2/telethonin complex is about 50 kDa and thus beyond the threshold of the renal filtration (in the range of 40-50 kDa) [21]. The protein complex is highly stable in plasma. Therefore, the Z1Z2/telethonin complex can be used as a scaffold to increase the stability of therapeutic peptides and proteins by genetically encoded fusion. In previous biochemical and structural studies, the Z1Z2 and telethonin proteins were expressed separately and then mixed to form the complex. However, telethonin was only found in inclusion bodies [9] rendering this not favorable for biomedical applications. To optimize and further simplify the purification of Z1Z2/telethonine various strategies have been explored here. In addition to improvement in refolding and oxidation sensitivity, coexpression and single-chain expression strategies are explored. We show that a single-chain construct comprising two Z1Z2 regions flanking an N-terminal fragment of telethonin that mediates Z1Z2 binding provides highly efficient expression of a stable fusion protein, termed ZTZ. The ZTZ protein will be beneficial for further research on therapeutic applications of the Z1Z2-telethonine complex.

2. Materials and methods

2.1. Strains and plasmids

All plasmid were constructed in *E. coli* strain DH5α. The sequences of all cloned PCR products and mutants were confirmed by sequencing (Invitrogen, Beijing, China). *E. coli* BL21(DE3) was used as the expression host. The expression plasmids pET-3d, pET-6d, pET-24d and pACYC-9d were obtained from Novagen (Madison, WI). The pACYCM-9d was modified from pACYC-9d by removing the His-tag. Primers were ordered from Sangon Biotechnology (Shanghai, China). Isopropylthio-β-D-galactoside (IPTG) was purchased from Sigma-Aldrich (Shanghai, China).

A construct comprising the two N terminal Ig domains of titin (residues 1-196) Z1Z2 was cloned into pET-3d and pET-6d vectors. In pET-3d, Z1Z2 is fused to the His_6-tag directly. In pET-6d, a TEV protease cleavage site exists between the His_6-tag and the Z1Z2 gene. Different regions of telethonin (residues 1-167 and residues 1-90) and cysteine mutants were cloned into pET-24d, pACYC-9d and pACYCM-9d.

2.2. Site-directed mutagenesis

The QuickChange™ site-directed mutagenesis protocol (Stratagene, La Jolla, CA) was used to introduce point mutations into telethonin$_{1-167}$ (C38S, C57S, C127S) using mutagenic oligonucleotides of 30 to 35 bases in length (Sangon Biotechnology, Shanghai, China). C8 and C15, which are close to the N-terminus of telethonin, were mutated into serines by PCR. A 49-mer primer with two point mutations and an N-terminal cloning site (NcoI) (5'-ATCCATGGCTACCTCAGAGCTGAGCAGCAGCGAGGTGTCGGAGGAGAAC-3') and the C-terminal cloning primer (5'-AAAGGTACCTTAGCCTCTCTGTGCTTCCTGG-3') with a KpnI restriction site were designed for the point mutation of C8 and C15. The PCR fragment was cut by the restriction enzymes NcoI and KpnI and cloned into the expression vectors pACYC-9d. The telethonin with no cysteine was referred to telethonin$_{1-167M}$, which was different from the telethonin$_{1-167}$ with 5 cysteines. The N-terminal truncation of telethonin$_{1-90}$ without cysteins was produced by PCR using the primer-F (CGCCATGGCTACCTCAGAGCTG) containing a NcoI restriction site and primer-R (TCGGATCCGGTACCTTACGGCA) containing a BamHI restriction site. The PCR fragment was cut by the restriction enzymes NcoI and BamHI, and cloned into the expression vectors pACYC-9d and pET-24d.

2.3. Expression and purification

The plasmids containing the genes encoding the Z1Z2 and telethonin$_{1-167}$ with a N-terminal His$_6$-tag were transformed into *E.coli* BL21 (DE3). Single colony was picked randomly and cultured in LB medium with 50 μg/ml kanamycin or 100 μg/ml ampicillin overnight at 37℃. For the co-expression, different pairs of the plasmids were co-transformed into *E. coli* BL21(DE3) and were grown in LB medim with 100 μg/ml ampicillin and 50 μg/ml kanamycin. Overnight cultures were grown in LB medium at 37℃, diluted 50-fold, and grown until the an optical density of 0.4-0.6 at 600 nm was reached. Then IPTG was added with a final concentration 1 mM and the bacteria were cultured additional 6 h at 30℃. The cells were harvested by centrifugation at 5000 rpm for 20 min at 4℃, resuspended in lysis buffer (25 mM Tric/HCl, 300 mM NaCl, pH 8.0, supplemented with 1 mg/ml lysozyme and 0.01 mg/ml DNase I), and lysed by pulsed sonication (5 min, 40% power, large probe, Fisher Scientific model 550) followed by centrifugation at 15000 rpm for 1 h.

Z1Z2 or Z1Z2/telethonin complexes were expressed in the soluble fraction. The supernatants of the lysates was applied to Ni^{2+}-NTA (Qiagen, Valencia, CA), which was pre-equilibrated with lysis buffer. The column was washed with the wash buffer (25 mM Tris/HCl, 300 mM NaCl, 30 mM imidazole, pH 8.0). Then the bound proteins were eluted with the elution buffer (25 mM Tris/HCl, 300 mM NaCl, 400 mM imadazole, pH 8.0). The eluted fractions were dialyzed against 25 mM Tris/HCl , pH 8.0 for further treatment. In order to obtain the pure proteins, after the dialysis, the sample was applied to a MonoQ ion exchange column (10/100 GL, GE healthcare) pre-equilibrated with 25 mM Tris/HCl, pH 8.0.

The telethonin was only expressed into inclusion bodies. After lysis and centrifugation, the pellets were resuspended in denaturing buffer (25 mM Tris/HCl, 8 M urea, pH 8.0). After the second centrifugation, the supernatant containing denatured telethonin, was applied to Ni^{2+}-NTA column which was pre-equilibrated with the denaturing buffer. Then the column was

washed and eluted respectively with the wash buffer (25 mM Tris/HCl, 300mM NaCl, 30 mM imidazole, 8 M urea, pH 8.0) and the elution buffer (25 mM Tris/HCl, 300mM NaCl, 400 mM imadazole, 8 M urea pH 8.0).

2.4. Refolding telethonin in the presence of Z1Z2

The Z1Z2 and telethonin$_{1-90}$ with a N-terminal His$_6$-tag were separately expressed and purified as described above. The Z1Z2/telethonin$_{1-90}$ complex was formed by adding telethonin$_{1-90}$ solutions diluted to 4 M urea concentration into the same volume of the purified Z1Z2. The protein mixture containing Z1Z2 and telethonin$_{1-90}$ was dialyzed against 5 liters of dialysis buffer A (25 mM Tris/HCl, 500 mM NaCl, pH 8.0) for 3 h, and then against 5 liters of dialysis buffer B (25 mM Tris/HCl, pH 8.0), overnight. The dialysates were applied to a MonoQ ion exchange column (10/100 GL, GE healthcare) pre-equilibrated with 25 mM Tris/HCl, pH 8.0. The protein was eluted by a salt gradient of 0-1 M NaCl/100ml in the buffer (25 mM Tris/HCl, pH 8.0).

2.5. Expression of a Z1Z2-telethonin-Z1Z2 single chain fusion protein

The two molecules of Z1Z2 and one molecule of telethonin (residues 1-90) were linked to one polypeptide chain. The new protein was named ZTZ protein. The pET-3d containing the Z1Z2 with a N-terminal His-tag and a TEV protease cleavage site and the pET-24d containing the telethonin$_{1-90}$ were used as template for PCR. The primer pairs Ti-F1/Ti-R1 and Ti-F2/Ti-R2 (Table 1) were used to produce the flanking N-terminal and C-terminal part of Z1Z2, respectively. The primer pair Te-F/Te-R (Table 1) was used to produce the telethonin part in the middle of the polypeptide chain. The full-length of the ZTZ was obtained by overlap PCR used the primer pair Ti-F1/Ti-R2 and the mixture of the above three PCR production. The final full length of PCR production was cut by the restriction enzymes XbaI and BamHI, and cloned into the expression vectors pET-24d. The expression and purification of the ZTZ protein was carried out in the same way as the soluble Z1Z2, described above.

Table 1 Primers for the ZTZ single chain protein

Primer	Sequence
Ti-F1	CAGCTCTAGAAATAATTTTGTTTAA
Ti-R1	CGGGGTACCCGGAGCAGAACCAGAACCAGAACCTTGAACCAGTAATTCAGC
Te-F	TCTGCTCCGGGTACCCCGGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTTCTATGGCTACCTCAGAGCTG
Te-R	GGTCGGAGAACCAGCCGGACCAGAAGCACCCGGCAGTACCCGCTGG
Ti-F2	CCGGCTGGTTCTCCGACCGGTTCTGGTCCGGGTTCTGCTGGTTCTGGTCCGGGTTCTGCTGGTATGGCCACTCAAGCACC
Ti-R2	ATTCGGATCCGGTACCTTAA

Note: the nucleotides underlined denote the XbaI and BamHI restriction sites.

3. Results and discussion

3.1. Expression and purification of Z1Z2 and telethonin

The Z1Z2 and telethonin$_{1-167}$ constructs were cloned into pET-3d and pET-24d vectors respectively, resulting in the expression vectors pET3d-Z1Z2 and pET24d-telethonin$_{1-167.}$ The plasmids containing N-terminal His-tag and TEV cleavage sites were separately transformed into *E.coli* BL21 (DE3). Expression test showed that the Z1Z2 was expressed into the soluble fraction, while the full-length telethonin protein was only found in inclusion bodies (Fig. 1). Various optimization strategies, such as bacterial culture growth at lower temperature, cloning into a low copy number expression vector (pACYC-9d), co-expression with different chaperones, and fusion with different protein tags like Glutathione S-transferase (GST), Maltose binding protein (MBP), Calmodulin and etc., were tried to resolve the solubility problem of telethonin but failed (data not shown). The refolding protocol for telethonin yielded unstable protein in solution where telethonin tends to aggregate and precipitate[9].

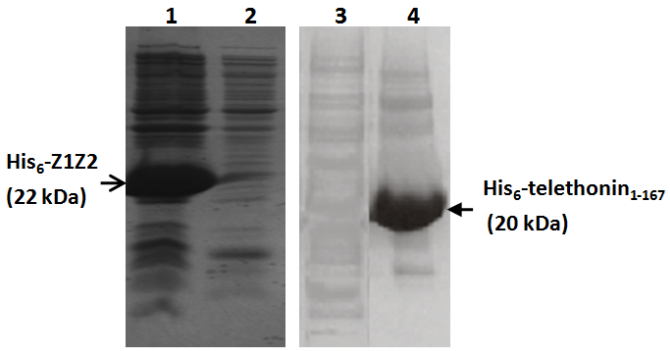

Fig. 1. SDS-PAGE analysis on the expression of Z1Z2 and telethonin. Z1Z2 and telethonin were expressed in *E. coli* BL21 (DE3) respectively. The supernatant of Z1Z2 (1), the inclusion body of Z1Z2 (2), the supernatant of telethonin (3) and the inclusion body of telethonin (4) were analyzed on 15% SDS-PAGE. The molecular weight of the proteins are indicated by the arrows.

3.2. Co-expression of telethonin$_{1-167}$ and Z1Z2

Based on biochemical data and the crystal structure telethonin forms a complex with the N-terminal two Ig domains (Z1Z2) of titin [5]. Complex formation can, however, not be achieved by simply mixing the two components, Z1Z2 and telethonin. Z1Z2 free in solution samples semi-extended conformations with close-hinge arrangements [15], but adopts a wide hinge opening conformation in the complex with telethonin [9]. The closed conformation of free Z1Z2 presumably explains why simply mixing of the two components does not allow complex formation [4, 15]. Therefore, coexpression of Z1Z2 and telethonin was considered and several pairs of co-expression vectors were designed: a), pET-3d-Z1Z2 and pACYC-9d-Telethonin; b), pET-6d-Z1Z2 and pACYC-9d-Telethonin; c), pET-3d-Telethonin and PACYCM-9d-Z1Z2; d), pET-6d-Telethonin and pACYCM-9d-Z1Z2.

Four pairs of co-expression vectors, which encode Z1Z2 and telethonin, respectively, were co-transformed and expressed. The supernatants of the lysis fraction were analyzed by SDS-

PAGE. Z1Z2 could be well over-expressed in all four expression vectors. In contrast, the expression of telethonin was dependent on the expression vectors. Telethonin was not expressed with pET3d or pET6d when Z1Z2 was expressed from pACYC9d (Fig. 2). As telethonin is not very soluble stabilization by the presence of Z1Z2 upon coexpression should enhance its solubility and expression. Therefore, upon expression of telethonin sufficient amounts of Z1Z2 should be present so that the complex can be formed. The pET series are high copy number expression vectors while pACYC has low copy numbers. If telethonin and Z1Z2 are expressed from pETs and pACYCs respectively, the expression speed of Z1Z2 is much lower than of telethonin. Thus, when telethonin is expressed insufficient amounts of Z1Z2 are present leading to precipitation of newly synthesized telethonin. After the expression of the complex was repeated several times, it was found that the ratio of the complex was not always shown as Fig. 2a,2b . The expression level of the complex was sometimes much lower, in contrast huge amount of free Z1Z2 were obtained. Both pETs and pACYCs vectors are controlled by the same T7 promoter. It is thus not possible to vary the concentration of inducers to adjust the expression level of the proteins differentially. Also, the pET and pACYC vectors used for co-expression have different antibiotic resistances and thus different concentration of antibiotics will influence the transcription level. Therefore, different concentrations of the antibiotics were applied to control the expression level of the proteins. In order to adjust the expression level of Z1Z2, a lower concentration of Ampcillin, 50 µg/ml instead of 100 µg/ml, was used in the co-expression experiments to achieve high yield of the complex. Notably, the expression speed of Z1Z2 was decreased to the level which was comparable to telethonin so that the complex could be formed according to the certain ratio of Z1Z2 and telethonin.

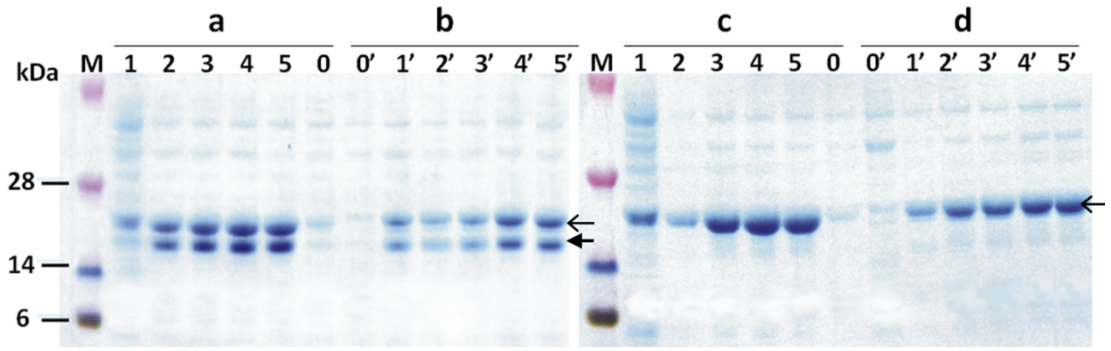

Fig. 2. SDS-PAGE analysis on the co-expression of Z1Z2 and telethonin: The different pairs of co-expression constructs: a) pET3d-His$_6$-Z1Z2 + pACYC9d-His$_6$-telethonin$_{1\text{-}167}$; b) pET6d-His$_6$-TEV-Z1Z2 + pACYC9d-His$_6$-telethonin$_{1\text{-}167}$; c) pET3d-telethonin$_{1\text{-}167}$ + pACYC9d-His$_6$-Z1Z2; d) pET6d-His$_6$-TEV-telethonin$_{1\text{-}167}$ + pACYC9d-His$_6$-Z1Z2 were co-transformed into *E. coli* BL21(DE3) and expressed. The supernatants of the lysates of the cultures incubated at 37℃ for 1 (1/1'), 2 (2/2'), 3 (3/3'), 4 (4/4') and 5 hrs. (5/5') after induction by IPTG were analyzed on 15% SDS-PAGE. The bands of expressed Z1Z2 and telethonin are indicated by the arrows.

3.3. Instabilities of telethonin

Wild type telethonin$_{1\text{-}167}$, harbors five cysteines (Cys), Cys8, Cys15, Cys38, Cys57 and Cys128,

rendering telethonin very sensitive to oxidation. Under non-reducing conditions telethonin can be completely oxidized within hours, even when bound to Z1Z2. Even in reducing conditions, i.e. by the addition of reducing agents, such as DDT and β-mercapto-ethanol; the addition of EDTA and flushing of buffers with nitrogen to remove oxidants, the purified complex of Z1Z2/telethonin$_{1-167}$ stored at 4℃ was still gradually oxidized. After 30 days, the telethonin was completely oxidized and its band on SDS SDS-PAGE was shifted from 20 kDa to the high molecular weight (Fig. 3). This high molecular band of telethonin could converted to the monomer by 10 mM TCEP. The sulfhydryl groups of cysteines in peptides and proteins were very easy to be oxidized by oxidants [22], resulting in the formation of disulfide bonds in intramolecular or intermolecular. The process of oxidation may occur in many steps, such as the protein purification, the sample storage and even on SDS-PAGE, generating different bands on SDS-PAGE with high molecular mass [23].

The oxidation of telethonin hampered the proceeding of purification. Therefore, site-directed mutagenesis was carried out to mutate the Cys8, Cys15, Cys38, Cys57 and Cys128 into serines, resulting in the telethonin$_{1-167M.}$ It has been reported that the cysteine-serine mutants of telethonin will have no influence on its complex formation with Z1Z2 [9]. To determine whether the mutations influence the complex formation His$_6$-telethonin$_{1-167M}$ and His$_6$-Z1Z2 were co-expressed.

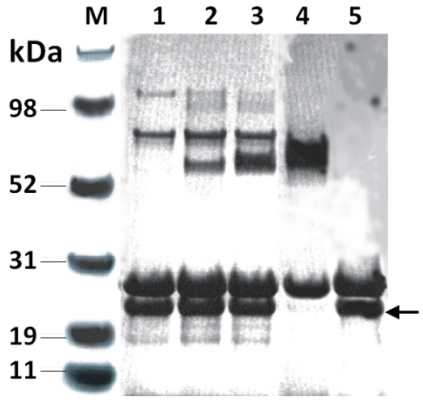

Fig. 3. The oxidation of telethonin. The purified complex of Z1Z2/telethonin$_{1-167}$ was stored at 4℃ for (1) 0 ; (2) 2 ; (3) 7 and (4) 30 days. The sample (4) was added 10 mM sulfhydryl reductant tris(2-carboxyethyl)phosphine (TCEP). The aliquots of the samples were mixed with the SDS-PAGE loading buffer without reducing agent and analyzed on 15% SDS-PAGE. The protein molecular marker (M) was used. The band of the telethonin monomer was indicated by the arrow.

The mutant of telethonin$_{1-167}$ and Z1Z2 were co-expressed and the complex was purified as described above. As expected no oxidation of telethonin in the complex was observed.

However, the full length telethonin in the complex was also found to be very sensitive to proteolysis. Protease inhibitor cocktails have to be used to prevent telethonin from proteolysis during purification. The purified complex was stored at 4℃ until 30 days without the protection by protease inhibitors. It was found that the telethonin$_{1-167}$ was degraded, while the

band of Z1Z2 remained intact. After storage at 4℃ just for hours, the telethonin start to be obviously degraded; after 7 days the 20 kDa band corresponding to the telethonin was degraded to 10 kDa (Fig. 4A). The 10 kDa band of telethonin was cut and sent to the measurement on mass spectrometry. The band was indentified to be the N-terminus of telethonin, corresponding to the sequence of 1-90 amino acid.

Previous studies have found that the N terminal region of telethonin is sufficient for binding to titin Z1Z2 [9]. Therefore an N terminal construct (residues 1-90) of telethonin was designed and the telethonin$_{1\text{-}90}$ was co-expressed with Z1Z2. Telethonin$_{1\text{-}90}$ formed a stable complex with Z1Z2 and the purified complex was stored at 4℃ until 30 days without any signs of degradation or oxidation (Fig.4B). This stable complex is thus of excellent quality for further structural and biochemical studies.

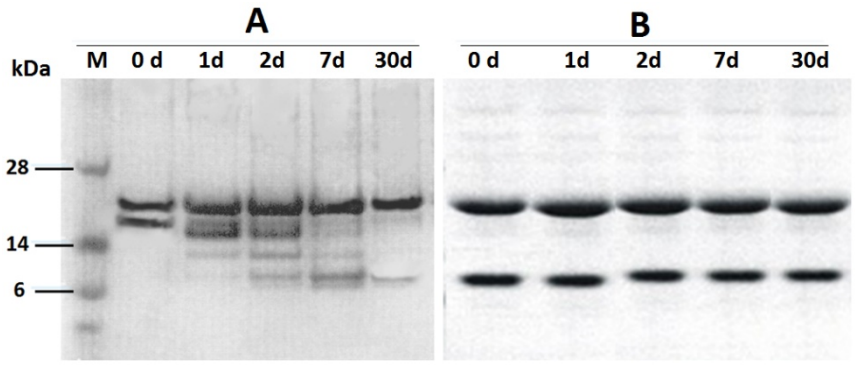

Fig. 4 The degradation of telethonin. The purified Z1Z2/telethonin$_{1\text{-}167M}$ and Z1Z2/telehonin$_{1\text{-}90}$ complexes in 25 mM Tris/HCl were placed at 4℃ for 30 days. Then a part of the complexes were taken out at specific intervals (0 d, 1 d, 2 d, 7 d and 30 d) and mixed with SDS-loading buffer followed by heating in the boiling water for 5 min and storing at -20℃ prior to analyze the stability of complex by 15% SDS-PAGE.

3.4. Refolding telethonin$_{1\text{-}90}$ in the presence of Z1Z2

We next tested if the minimal complex could be prepared by mixing of separately expressed Z1Z2 and telethonin$_{1\text{-}90}$. For this Z1Z2 was purified from the supernatant, however, the telethonin$_{1\text{-}90}$ needed to be purified from the inclusion bodies. Telethonin$_{1\text{-}90}$ was dissolved in 8 M urea. As described above, the complex does not form by simply mixing Z1Z2 and telethonin under the native condition. However, the complex could be reconstituted by adding Z1Z2 into the same volume of telethonin$_{1\text{-}90}$ solution which was previously diluted to a concentration of 4 M urea, and subsequently dialyzed first against high salt buffer (25 mM Tris/HCl, 500 mM NaCl, pH 8.0), and then against buffer without salt (25 mM Tris/HCl, pH 8.0). If Z1Z2 and telethonin were mixed at high concentration of urea (>4 M) and dialyzed, most of the telethonin immediately precipitated and no complex was formed. Upon mixing of Z1Z2 and telethonin at 2 M urea followed by immediate dialysis against buffer without salt precipitation of telethonin was observed. Z1Z2 preferentially presents semi-extended conformations in solution, which are not suitable for binding [15]. At the condition of 2 M urea, Z1Z2 is partially

denatured and the close-hinge confirmation of Z1Z2 could be opened; while telethonin is half-refolded and remains soluble in solution. When the solution of Z1Z2 and telethonin is dialyzed against the buffer without the denatured regent, the conformations of Z1Z2 and telethonin are induced upon binding. The high concentration of salt will keep telethonin soluble until it forms the complex with Z1Z2.

Z1Z2 and the complexes were analyzed on SDS-PAGE and Native-PAGE (Fig. 5). Z1Z2 run much slower than the complex on Native-PAGE. It seemed that Z1Z2 formed oligomer on the Native-PAGE. But Z1Z2 behaved like a monomer on size exclusion chromatography (Fig. 6). The previous result showed that the oligomerization of Z1Z2 on Native-PAGE could be interrupt by adding EDTA or EGTA into the running buffer [9]. It suggested that Z1Z2 could form the oligomer in the presence of metal ions, like calcium. But it was interesting that Z1Z2 in solution always behaved like monomer. It still remains unclear.

For crystallization and structure determination of the Z1Z2/telethonin complex [5] refolding telethonin$_{1\text{-}90}$ in presence of Z1Z2 was performed. The amount of Z1Z2 was kept constant and different concentrations of telethonin were added to form the complex with Z1Z2. As shown in Fig. 6 that complex formation gradually increases with the increase of Z1Z2 protein. As the concentration of telethonin reaches 0.5, even 2 ratio of Z1Z2, there is still Z1Z2 left, which did not form complex. This indicates that not every molecule of telethonin could form the complex with Z1Z2 and thus excessive amounts of telethonin need to be added for complex formation. After dialysis, the excesss of telethonin will precipitate and can thus easily removed from the mixture.

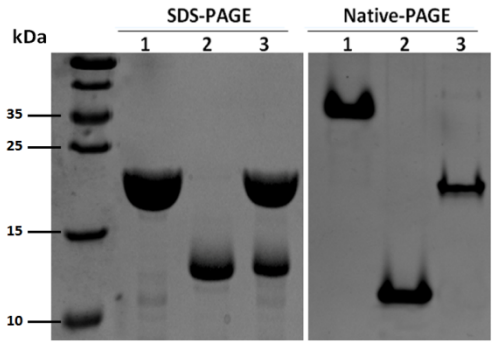

Fig. 5. SDS-PAGE and Native-PAGE analysis: 1) His$_6$-Z1Z2; 2) His$_6$-telethonin$_{1\text{-}90}$ and 3) the complex were analyzed on 15% SDS and Native-PAGEs.

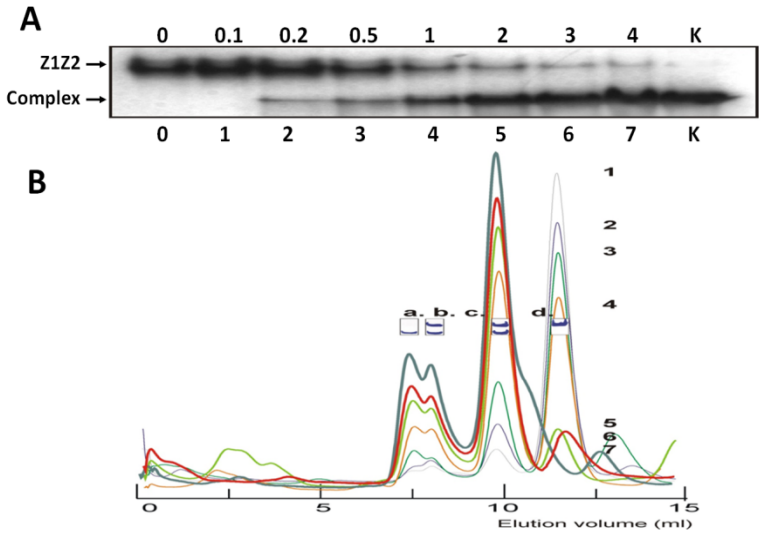

Fig. 1Z2/telethonin complex formation: The different ratio of telethonin were refolded in the presence of Z1Z2. The ratios of telethonin/Z1Z2 were indicated as 1) 0.1, 2) 0.2, 3) 0.5, 4) 1, 5) 2, 6) 3 and 7) 4. The complex mixtures together with 0) Z1Z2 and K) the purified complex as control were analyzed on the Native-PAGE (A) and size exclusion chromatography (B). The chromatography was carried out on the Superose 6 column (10/300 GL, GE healthcare) previously equilibrated with the buffer (20 mM Tris/HCl, pH 8.0, 100 mM NaCl). The fraction samples from each peak corresponding to the aggregation fractions (a, b), the complex (c) and Z1Z2 (d) were analyzed by SDS-PAGE.

3.5. Z1Z2/telethonin$_{1-90}$ complex was expressed on the single peptide chain

To further simplify the purification step and facilitate the application of the Z1Z2/telethonin$_{1-90}$ complex as a scaffold to prolong the half-life of the peptides or proteins by genetically fusion, two linkers was designed to connect the Z1Z2, telethonin$_{1-90}$ and Z1Z2 by end to end manner. Thus the complex could be expressed and purified on the single peptide chain, denoted as ZTZ protein. The flexible linkers inserted between the first Z1Z2 and telethonin$_{1-90}$, and between telethonin$_{1-90}$ and the second Z1Z2 were SGSGSAPGTPGGGGSGGGGSGGGGS and GASGPAGSPTGSGPGSAGSGPGSAG, respectively. The ZTZ protein was expressed mainly in the soluble fraction (Fig.7A, lane4) and was easily to be purified by the Ni^{2+}-NTA column (Fig. 7A, lane5). The eluted fraction was subjected to His-tag cleavage using the TEV protease, and then applied to Ni^{2+}-NTA column to get the pure ZTZ protein with a molecular weight about 60 kDa (Fig.7A lane 6). The ZTZ protein was further purified using the Q ion exchange column by linear gradient elution (from 0 to 300 mM NaCl) to get the high purity ZTZ protein. And the ZTZ protein was eluted at 156 mM NaCl (Fig.7B). The ZTZ protein was expressed in the soluble fraction and easily to be further purified by Ni^{2+}-NTA and Q ion exchange column.

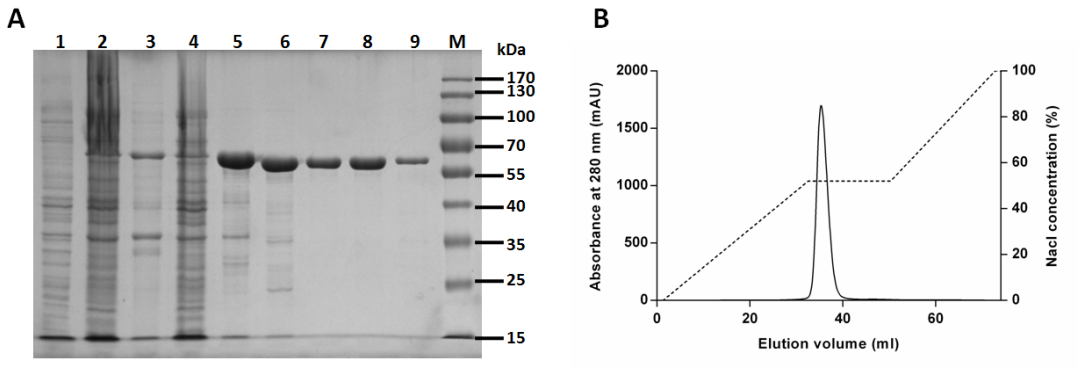

Fig. 7. Expression and purification of the ZTZ protein. (A) 10% SDS-PAGE analysis of the expression and purification of ZTZ protein. 1) whole cell lysate before IPTG induction (non-induced cell lysate); 2) whole cell lysate induced by 1 mM IPTG at 30℃; 3) inclusion body dissolved by 8 M Urea; 4) supernatant; 5) eluate fraction from Ni^{2+}-NTA column using elution buffer (25 mM Tris/HCl, 300 mM NaCl, 400 mM immidazole, pH 8.0); 6) flow through fraction from Ni^{2+}-NTA column after cleavage of the His-tag by the TEV protease; 7-9) eluate fraction from the Q ion exchange column; M) molecular weight marker. (B) Purification of the ZTZ protein using Q ion exchange column by linear gradient (from 0 to 300 mM NaCl). The ZTZ protein was eluted at 156 mM NaCl (52%).

4. Conclusions

Here, we show that soluble telethonin can be obtained successfully by co-expression from two vectors. The vector selection for the different binding partners of the complex is very important for the successful formation of the complex. In general, the soluble partner was cloned on to the high copy number of the vector, on contrary the insoluble one to the low copy number of the vector. Sometimes the different concentrations of antibiotics could be used as pressure to adjust the expression level of the binding partners to achieve high yield of the complex. The oxidation and proteolysis problem of telethonin could be resolved by the mutation of cysteines into serines and C-terminal truncation. These alterations on telethonin had no influence on the complex formation. The complex could be formed by refolding telethonin in the presence of Z1Z2. The mixture of Z1Z2 and telethonin was diluted to the concentration of 2 M urea. Under this condition, Z1Z2 was half-denatured, telethonin partially folded and the complex initially formed. With additional dialysis against the buffer with high concentration of the salt, the complex was obtained.

In order to develop a protocol that enables efficient and reliable expression and purification of large amounts, the ZTZ fusion protein was designed. The expression as a single chain yields soluble protein, greatly facilitating further purification. The successfully purified ZTZ protein is a promising and useful sample for application as a scaffold to improve the pharmacokinetics and pharmacodynamics of peptide and protein drugs. The strategies for extending half-life of therapeutic peptides and proteins have been made great progress with the development of the biotechnology and genetic engineering. Genetically fusion of the

peptides and proteins to a larger protein or the unstructured polypeptide, such as XTEN, PAS and HAP etc., to reduce the renal clearance rate and lower the hydrolysis of enzymes via steric hindrance has been proven to be an effective way to prolong the half-life of the peptides and proteins [20]. The ZTZ protein is about 60 kDa excess to the threshold of renal filtration [21], and is anti-enzymatic degradation in plasma. Therefore, the ZTZ protein can be used as a carrier to extend the half-life of the peptides or proteins by genetically fusion to the N-terminus or C-terminus. The ZTZ protein expressed in the soluble fraction and purified easily has great potential in the application of the peptides and proteins in the clinical treatment. The further research will be conducted in the future.

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Highlights

1) Several strategies were established to obtain the soluble telethonin.

2) A protocol was established to obtain the soluble Z1Z2/telethonin complex.

3) The mutation and truncation of telethonin made Z1Z2/telethonin complex super stable.

4) The Z1Z2/telethonin complex was successfully expressed on the single peptide chain.