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Original Article

Structural features and thermal stability of shrimp 14-3-3ε

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Abstract

In our previous study, we reported that the nucleotide sequence of a 14-3-3ε cDNA clone from *Litopenaeus vannamei* encodes two transcripts, designated as 14-3-3EL and 14-3-3ES. Based on the retained introns we observed in 14-3-3EL by alternative splicing, we aimed to investigate the genomic structure of this gene. The 14-3-3ε gene, encoded by 2,311 bp of DNA sequence, is interrupted by three introns of 48, 1,124 and 365 bp in size. In addition, using the SWISS-MODEL, the predicted 3D model of 14-3-3EL has twelve α-helices and two β-sheets, whereas 14-3-3ES has only eleven helices in its tertiary structure. Additionally, we generated the 14-3-3EL and 14-3-3ES proteins and measured their thermal stabilities. The results demonstrated that the quantity of the 14-3-3EL protein decreased significantly at all temperatures tested. In contrast, the quantity of the 14-3-3ES protein remained unchanged, suggesting that 143-3-3EL is less stable than 14-3-3ES.

Keywords: alternative splicing, 14-3-3 epsilon, β-sheet, genomic structure, protein stability

1. Introduction

Alternative splicing is one of the most important mechanisms that allows for the production of a variety of different protein isoforms from only one gene (Keren, Lev-Maor, & Ast, 2010; Rodriguez-Martin *et al.*, 2005). Gene regulation through alternative splicing is more versatile than regulation through promoter activity. Variant transcripts generated through alternative splicing, similar to those initiated from distinct promoters, are often tissue- and/or development-specific, driving effects observed only in certain cells or developmental stages (Stamm *et al.*, 2005). Alternative splicing contributes to the RNA processing itself, from pre- to post-transcriptional events. Thus, alternative splicing has a role in almost every aspect of protein function, including binding between proteins and ligands, nucleic acids or membranes, localization, enzymatic properties and protein stability (Nilsen & Graveley, 2010; Stamm *et al.*, 2005; Wang *et al.*, 2015).

14-3-3 epsilon (14-3-3ε) belongs to the 14-3-3 protein family, which is a class of highly conserved 28-33 kDa acidic polypeptides involved in regulating signal transduction pathways, apoptosis, cell-cycle control, cellular proliferation, differentiation, stress response and survival (Bridges & Moorhead, 2005; Fu, Subramanian, & Masters, 2000). Among all 14-3-3 proteins, 14-3-3ε is the isoform most associated with alternative splicing. Alternatively spliced forms of 14-3-3ε exhibit distinct abilities, ranging from a complete loss of function to subtle effects that are difficult to detect. For example, in rainbow trout, the alternatively spliced form of

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14-3-3E1ΔC17, lacking 17 amino acid residues at the C terminus, displays tissue-specific as well as stage-specific expression during ovarian development and early embryogenesis, whereas the wild-type 14-3-3E1 (14-3-3E1wt) is ubiquitously expressed (Wanna, Rexroad, & Yao, 2010). In humans, the truncated splicing variant of 14-3-3 epsilon cannot dimerize; however, the 14-3-3 epsilon monomer can sufficiently protect cells from apoptosis after UV irradiation (Han *et al.*, 2010). Similarly, *Litopenaeus vannamei* have two alternative 14-3-3 epsilon isoforms, 14-3-3EL and 14-3-3ES, where the 14-3-3EL isoform contains an intronic insertion of 48 nucleotides. After white spot syndrome virus (WSSV) infection, the expression of 14-3-3EL mRNA increases significantly in the gill and muscle tissue of *L. vannamei*, but the expression of 14-3-3ES only increases in muscle tissue. Therefore, 14-3-3EL and 14-3-3ES might mediate different cellular processes (Wanna, Thipwong, Mahakaew, & Phongdara, 2012). To further elucidate the effects of alternative splicing in the shrimp 14-3-3ε, its genomic structure and protein stability were investigated in this study.

2. Materials and Methods

2.1 Isolation of genomic DNA

Genomic DNA was extracted from the muscle tissue of normal white shrimp using the phenol/chloroform and ethanol precipitation method. Muscle tissue (0.1 g) was chopped into small pieces and dissolved in 700 µl of extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 200 mM Sucrose, 50 mM EDTA pH 8.0, 1% SDS). Next, 10 mg/ml Proteinase K (Sigma, UK) and 1 mM DTT were added to a final concentration 0.3 µg/ml and 200 µg/ml, respectively, and the solution was incubated for 3 hrs at 55°C in a water bath with gentle agitation every hour. The extraction solution was centrifuged at 12,000 rpm for 1 min, and aliquots of the supernatant were transferred to 1.5 ml centrifuge tubes. RNase A (10 µg/ml) was added to a final concentration of 0.5 µg/ml and incubated at 37°C for 1 hr. The DNA was extracted two times with phenol/chloroform/isoamyl (25:24:1). After centrifugation, the DNA in the upper phase was precipitated in 2 volumes of absolute ethanol, gently mixed, and incubated at -70°C for 1 hr. The precipitated nucleic acid was pelleted and washed with 70% ethanol. The DNA pellet was air-dried and resuspended in 200 µl sterile water. DNA quality controls were performed using 1% agarose gel electrophoresis, and the DNA concentration was determined with a NanoDrop (ND-2000) spectrophotometer.

2.2 PCR amplification of a genomic region

Genomic PCR was performed using the advantage 2 polymerase mix (Clontech, USA). The primer pairs used to amplify 14-3-3ε are presented in Table 1. The primers 1433F-int1 and 1433R- int1 were used to amplify intron 1, the primers 1433F- int2 and 1433R- int2 were used to amplify intron 2, and the primers 1433F- int3 and 1433R- int3 were used to amplify intron 3. The genomic DNA from white shrimp muscle that was used as a template was incubated in advantage 2 polymerase mix for 1 cycle of 95°C for 2 min, 7 cycles of 94°C for 30 sec and 72°C for 3 min, 32 cycles of 94°C for 30 sec and 60°C for 3 min, and finally 1 cycle of 68°C for 10 min. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, cloned and sequenced.

2.3 Genomic structure prediction and protein modeling

The 3D structures were performed with the SWISS-MODEL (Panrat *et al.*, 2012). The 14-3-3 template structures were obtained from the PDB database (http://swissmodel.expasy.org). These were evaluated by Ramachandran plot analysis with ProFunc (http://www.ebi.ac.uk/thornton-srv/databases/profunc), and the Pro-origami server (Stivala, Wybrow, Wirth, Whisstock, & Stuckey, 2011) (http://munk.cis.unimelb.edu.au/pro-origami/porun.shtml) was used to identify the potential functions within the 3D structures. The best template was automatically selected based on multiple-threading simulations and used for the structural model.

2.4 Protein expression

The full length cDNA 14-3-3 epsilon of *L.vannamei* was obtained using primers 14-3-3εF-BamHI and 14-3-3εR-XhoI for amplify product of 822 bp 14-3-3EL and 774 bp 14-3-3ES. The excised inserts were subcloned in to pET vector (Novagen, USA) digested with *Bam*HI and *Xho*I. The

Table 1. Nucleotide sequences of the primers used in this study.

Fragment	Primer name	Primer sequence
Intron 1	1433F-int1	5'CGGTGAGGAGAAGCTGGAGATG3'
	1433R- int1	5'TCTCTGCAGCCGCCTTCCTGTC3'
Intron 2	1433F-int2	5'TCGCTTACAAGGCAGCCAGTGA3'
	1433R- int2	5'GCTGCATGATTAGGGTGGAGTC3'
Intron 3	1433F-int3	5'CCACACACCCCATCAGGCTGG3'
	1433R- int3	5'CTCGAGTTAGCTCACATCCTGGTCCTCC3'
14-3-3ε	1433εF-BamHI	5'GGATCCATGACGGACCGGGAGGACAAC3'
	1433εR-XhoI	5'CTCGAGGGAGGACCAGGATGTGAGCTAA3'

recombinant plasmids pET-14-3-3EL and pET-14-3-3ES in *E. coli* BL21 (DE3) were streaked onto LB agar plates containing 25 µg/ml kanamycin. Twenty milliliters of LB solution containing 25 µg/ml kanamycin was inoculated with a single colony and cultured at 37°C for 16-18 hrs. The starting culture was transferred into 200 ml of LB solution containing 25 µg/ml kanamycin and shaken at 37°C for another 1-1.5 hr. When the culture reached an OD_{600} of ~ 0.5, 0.1 mM IPTG was added, and the culture was incubated at 37°C for 3 hrs. The bacteria were harvested by centrifugation at 4,000 rpm for 20 min at 4°C and analyzed by 12% polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE).

2.5 Protein purification

The bacterial pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole and 2% (v/v) Triton-X 100; pH 8.0) containing 1 mM PMSF, 10 mg/ml lysozyme and 2 mg/ml *DNase*I. The protein lysates were purified using Ni-NTA beads (Invitrogen, USA), and the histidine-tagged fusion proteins were eluted with an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole). The eluted protein was analyzed by 12% SDS–PAGE and visualized using Coomassie blue staining. The protein concentration was assessed using the Bradford standard protocol.

2.6 Thermal stabilities of 14-3-3ε

Protein stability was investigated by incubating 14-3-3ES and 14-3-3EL at different temperatures. After protein purification, 4 µg of each protein were suspended in elution buffer to a final concentration of 0.27 µg/µl. The two purified proteins were incubated at 25, 30, 35, 40, 45, 50, 55 and 60°C for 1 hr. In addition, these proteins were stored at -20°C and -80°C for 1, 2, and 3 weeks. Proteins were analyzed by 12% SDS-PAGE and quantified with the Quantity One program (Bio-Rad, USA). The final results represent the mean of three experiments.

3. Results and Discussion

3.1 Genomic structure

L. vannamei 14-3-3ε was previously isolated and characterized, revealing the presence of two 14-3-3ε isoforms (Wanna *et al.*, 2012). To analyze the genomic structure of 14-3-3ε, we sequenced the 2,311 bp region encoding the shrimp 14-3-3ε gene and mapped the positions of its four exons (389 bp, 120 bp, 315 bp, and 58 bp), which are separated by 48 bp, 1,124 bp and 365 bp introns, respectively (Figure 1). This similar genomic structure was also observed for the *Drosophila* 14-3-3 epsilon gene (Voigt, Liebich, Wöstemeyer, Adam, & Marquardt, 2000), which harbors 4 exons interrupted by 3 introns. Both shrimp and *Drosophila* are arthropod, and they have similar Toll-like receptors (TLRs) pathway (Chen, Li, & He, 2014). In addition, *Drosophila* is a well-studied model organism in Biology. This study demonstrated that the insertion of 48 bp in 14-3-3EL occurred from intron 1 by alternative splicing.

3.2 Protein modeling

When we analyzed the predicted 3D structure of the two 14-3-3ε isoforms with the SWISS-MODEL, 14-3-3EL exhibited twelve α-helices, including the amino acid residues 39-71 (H3), part of the pseudosubstrate domain at residues

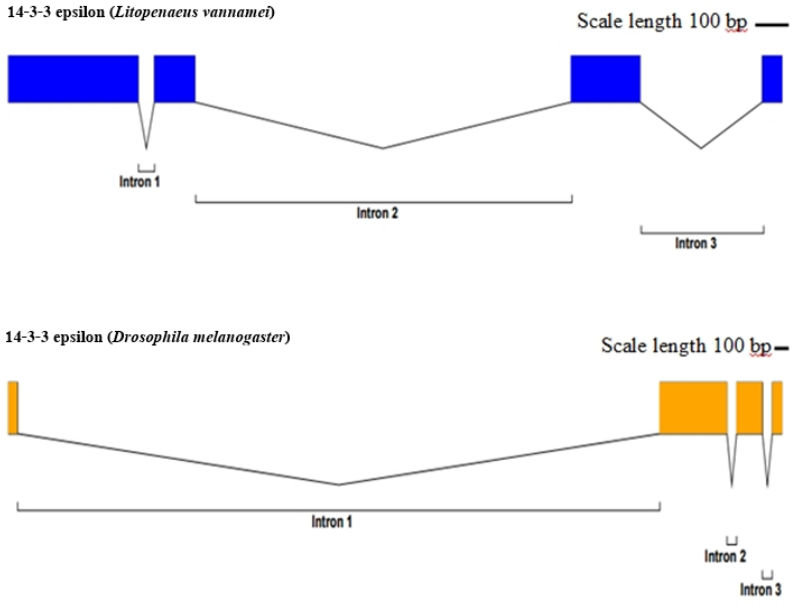

Figure 1. Physical structures of the 14-3-3 epsilon protein of the white shrimp, *L. vannamei* and *D. melanogaster.*

55-69, an annexin similarity domain at residues 125-157 that formed H6, S1, H7, S2 and H8, an EF hand motif at residues 225-235 between H11 and H12, two β-sheets and one β hairpin loop at residues 129-153 (Figure 2). The 3D structure analysis revealed that 14-3-3ES harbored only eleven α-helices, including the pseudosubstrate domain at residues 55-69 on H3, an annexin similarity domain at residues 125-141 between H6 and H7, an EF hand motif at residues 209-219 between H10 and H11 (Figure 3). Interestingly, two β-sheets caused by an insertion of 48 bp (intron 1). The conformational preferences of the protein are extremely important as it defines its function. In humans, the crystal structures of the 14-3-3 protein family members each revealed a 14-3-3 monomer consisting of a group of nine α-helices (Obsil & Obsilova, 2011; Yang *et al.*, 2006); thus, the α-helix structure is a common feature of the 14-3-3 protein family. The α-helix secondary structure is stabilized by strong hydrogen bonds, whereas β-sheets are more flexible than α-helices. From 3D structure prediction, β-sheets in 14-3-3EL were caused by an insertion of 48 bp in annexin similarity domain. These β-sheets formed the flexible loops in its 3D structure (Figure 3) and may affect to the stability and function of 14-3-3EL protein.

The effects of alternative splicing on the function of a single protein range from changes in the substrate or interaction partner specificity to the regulation of DNA-binding properties (Stetefeld & Ruegg, 2005). To change the function of a protein by alternative splicing, its structure may be changed accordingly.

3.3 Protein expression and stability

The alternative splicing of 14-3-3ε has been observed in several organisms (Han *et al.*, 2010; Wanna *et al.*, 2010,

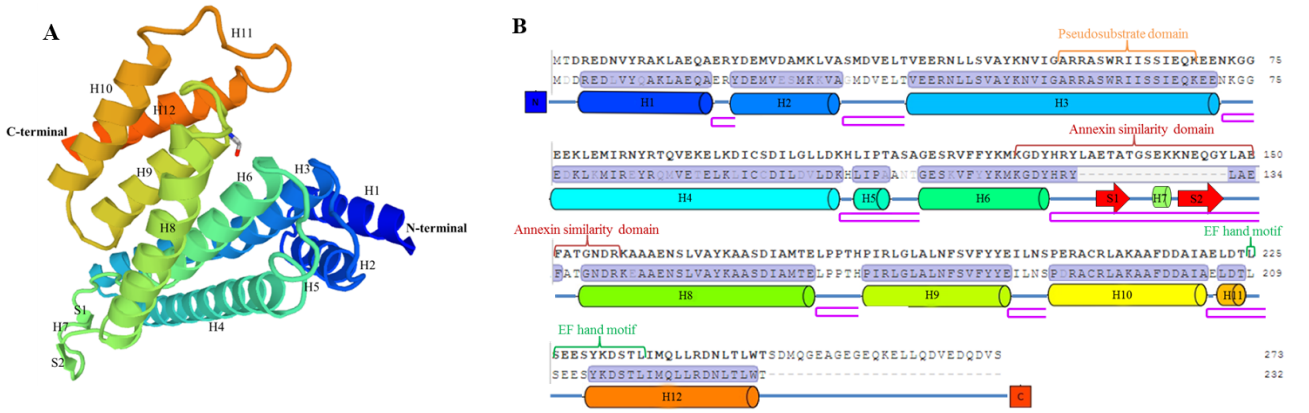

Figure 2. Predicted 3D model and 2D functional mapping of 14-3-3EL from *Litopenaeus vannamei*. **(A)** The predicted monomer structure of 14-3-3EL contains 12 α-helices and 2 β-sheets, with the β hairpin loop at residues 129-153. **(B)** A graphical representation of the functional mapping of the 14-3-3EL protein was analyzed by ProFunc and the Pro-origami server. The following elements are shown: the pseudosubstrate domain at residues 55-69, the annexin similarity domain at residues 125-157 and the EF hand motif at residues 225-235.

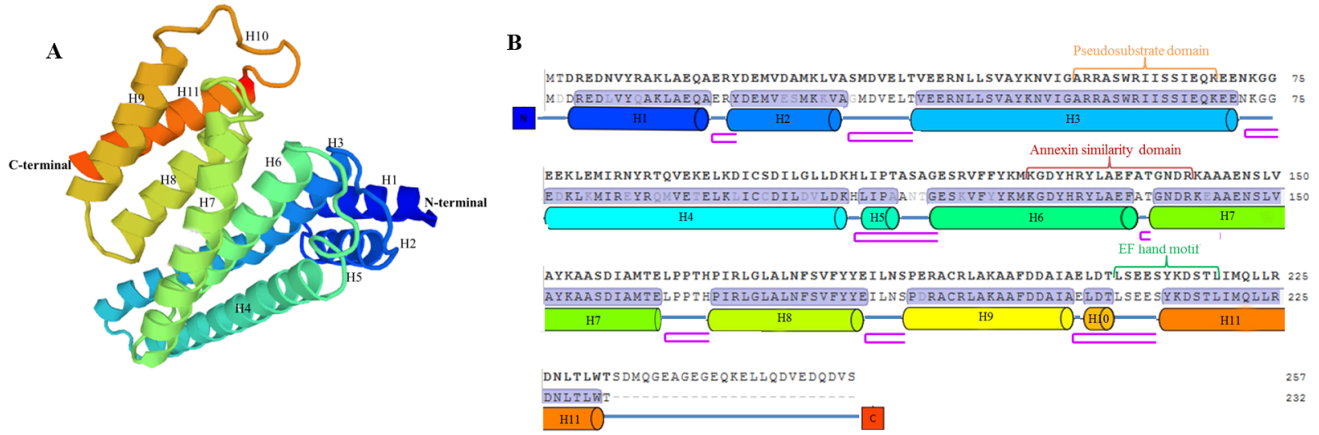

Figure 3. Predicted 3D model and 2D functional mapping of 14-3-3ES from *Litopenaeus vannamei*. **(A)** The predicted monomer structure of 14-3-3ES contains 11 α-helices. **(B)** A graphical representation of the functional mapping of the 14-3-3ES protein was analyzed by ProFunc and the Pro-origami server. The following elements are shown: the pseudosubstrate domain at residues 55-69, the annexin similarity domain at residues 125-141 and the EF hand motif at residues 209-219.

2012). In this study, we describe the effect of alternative splicing on the stability of 14-3-3ε proteins in *L. vannamei* under various temperatures. The expression levels of the His-14-3-3EL and His-14-3-3ES proteins increased significantly when IPTG was added to the LB culture with 25 µg/ml kanamycin to a final concentration of 0.1 mM. The His-14-3-3EL and His-14-3-3ES fusion proteins are approximately 36 kDa and 34 kDa, respectively, determined by 12% SDS-PAGE analysis. The target proteins could be expressed at levels 3-fold higher than the uninduced proteins, and the target proteins were observed both in a soluble and insoluble form (data not shown). The soluble 14-3-3ε proteins were purified using Ni-NTA beads, and a single major band was observed on 12% SDS-PAGE (Figure 4).

To determine whether the alternative splicing of shrimp 14-3-3ε affected protein stability, the two proteins were incubated at different temperatures (25, 30, 35, 40, 45, 50, 55, and 60°C) for 1 hr. Approximately 75% - 97% of the total 14-3-3ES protein remained, whereas approximately 61%-84% of the total 14-3-3EL was observed (Figure 5). In addition, when these two proteins were stored at -20°C and -80°C for 3 weeks, similar to the results at high temperatures, the level of the 14-3-3EL protein decreased significantly compared to 14-3-3ES levels (Figure 6). These observations suggest that the 14-3-3EL protein may be less stable than the 14-3-3ES protein. As mention above, the insertion of 48 bp resulted in β-sheet in 3D structure of 14-3-3EL whereas the wild type 14-3-3ES has only α-helices. The β-sheets are more flexible than α-helices. Thus, the stability decrease in 14-3-3EL at high and low temperature might be resulted from flexible β-sheet in its structure. Pace, Treviño, Prabhakaran, and Scholtz (2004) reported that after protein unfold in ethanol, it is often observed to refold into rod-like structure with high content of α-helix. Thus, α-helices are expected to be stable structure of proteins when they exposed to denature condition.

This study suggested that the alternative splicing with insertion of 48 bp may affect to stability of 14-3-3 epsilon protein. The effect of alternative splicing on protein stability has been studied for the *c fos* gene, where the alterative form is more stable than the wild type transcript (Nestler, Kelz, & Chen, 1999). In humans, an alternatively spliced thyroperoxidase (TPOzanelli) has a shorter half-life with attenuated expression at the cell surface compared to wild type (TPO1) (Niccoli-Sire, Fayadat, Siffroi-Fernandez, Malthierry, & Franc, 2001). Additionally, a 78 bp insertion into the caspase 3-sensitive site of protein kinase C resulted in a protein kinase C delta isoform insensitive to the protease (Sakurai, Onishi, Tanimoto, & Kizaki, 2001).

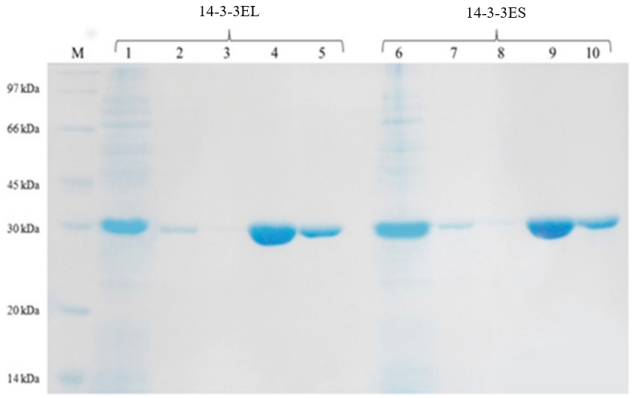

Figure 4. 12% SDS-PAGE analysis of 14-3-3EL and 14-3-3ES after His-tag purification. Lane M, protein molecular weight markers; Lanes 1 and 6, culture lysate before purification; Lanes 2, 3, 7 and 8, two successive washes of the columns; Lanes 4, 5, 9 and 10, two successive elutions showing the purified His-tagged 14-3-3EL and His-tagged 14-3-3ES proteins.

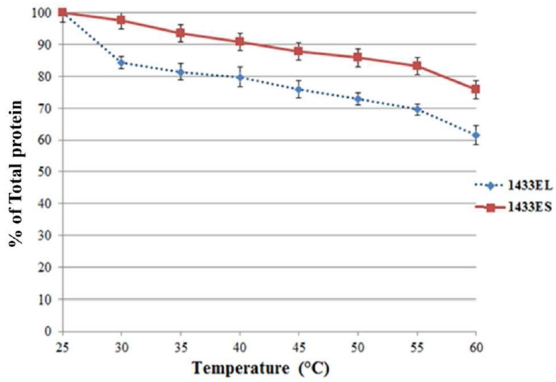

Figure 5. Analysis of the stability of the purified recombinant 14-3-3ε protein at various temperatures.

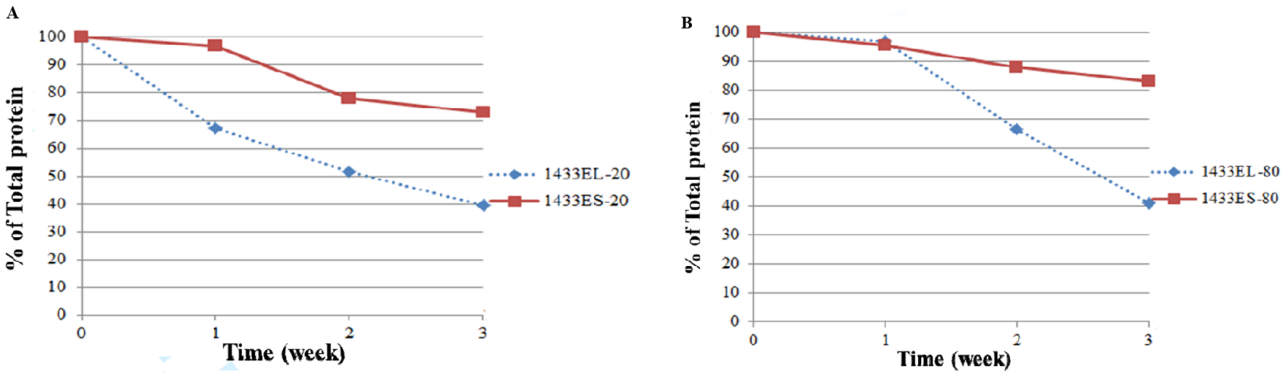

Figure 6. Remaining 14-3-3ε protein after storage at -20°C (A) and -80°C (B) for 3 weeks.

4. Conclusions

This study demonstrated that the alternative splicing of shrimp 14-3-3ε alters the protein conformation and leads to the expression of a less stable form. The expression of the alternative form might increase its functional variability, and the production of the unstable protein may protect the cell out from danger for a short time. However, the relationship between alternative splicing and the function of 14-3-3 epsilon proteins in shrimp remains unclear and requires further study.

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