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Research Paper

cDNA, genomic sequence and overexpression of crystallin alpha-B Gene (*CRYAB*) of the Giant Panda

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*a*B-crystallin, a small heat-shock protein, has been shown to prevent the aggregation of other proteins under various stress conditions. Here we have cloned the cDNA and the genomic sequence of *CRYAB* gene from the Giant Panda (*Ailuropoda melanoleuca*) using RT-PCR technology and Touchdown-PCR, respectively. The length of cDNA fragment cloned contains an open reading frame of 528bp encoding 175 amino acids and the length of the genomic sequence is 3189bp, containing three exons and two introns. Alignment analysis indicated that the nucleotide sequence and the deduced amino acid sequence are highly conserved to other four species studied, including *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Bos taurus*. The homologies for nucleotide sequences of Giant Panda *CRYAB* to that of these species are 93.9%, 91.5%, 91.5% and 95.3%, respectively, and the homologies for amino acid sequences are 98.3%, 97.1%,97.7% and 99.4%, respectively. Topology prediction shows that there are only four Casein kinase II phosphorylation sites in the CRYAB protein of the Giant Panda. The cDNA of *CRYAB* was transfected into *E. coli*, and the CRYAB fused with the N-terminally His-tagged protein gave rise to the accumulation of an expected 24KDa polypeptide, which accorded with the predicted protein. The expression product obtained could be used for purification and study of its function further.

Key words: cDNA cloning; *CRYAB*; the Giant Panda (*Ailuropoda melanoleuca*); genomic cloning; overexpression.

1. Introduction

The lens proteins consist of about 95% crystallins. The lens continues to grow throughout life, forming new fiber cells at the periphery of lens without ever replacing the older cells. As a consequence, the cells in the nucleus of the lens contain proteins that are as old as the animal itself. To warrant transparency, the light-scattering organelles, including the nuclei, are broken down in the later stages of differentiation [1-4]. The transparency and high refractive index of the normal lens is achieved by a regular arrangement of the lens fiber cells during growth of the lenticular body and by the high concentration and the supramolecular organization of the lens-specific proteins, the crystallins, within each fiber cell [5,6]. In the mammalian lens, 3 major classes of crystallins are distinguished: alpha, beta, and gamma. The largest, alpha-crystallins, which composed of 2 primary gene products--alpha-A and alpha-B, are some of the most abundant soluble proteins in the lens and, along with the other lens crystallins, play an important role in establishing and maintaining the optical properties of the lens [7]. *a*B-crystallin, an abundant eye lens protein, is also present in other tissues and is heat- and stress-inducible, where as *a*A-crystallin, the other eye lens protein, is not heat-inducible [8-11]. Both *a*A and *a*B-crystallin (subunitMolecular mass≈20 kDa) form homo- as well as hetero-multimers of various sizes [12] and exhibit molecular-chaperone-like activity in preventing aggregation of other proteins [13-16], with *a*B-crystallin being more efficient than *a*A-crystallin [15,16]. *a*-crystallins are associated with some type of cancers such as basal-like breast carcinomas [17], and belong to the sHsp (small heat-shock protein) family [18].

The Giant Panda (*Ailuropoda melanoleuca*) is a rare species currently found only in China. They are known as a "living fossil". For many years, studies on the Giant Panda have been mainly concentrated on fields such as breeding and propagation, ecology, morphology, taxology, physiology and pathological biochemistry. Recently, researches on genetic diver-

sity, parentage and phylogenesis *et al.* have been developed, while reports on functional gene are handful [19-27].

In the present study, we have amplified the cDNA sequence of *CRYAB* gene from the total RNAs extracted from the skeleton muscle of the Giant Panda, and then analyzed the sequence characteristics of the protein encoded by the cDNA and compared it with those of human and other mammalian species reported. The overexpression was also done in *E. coli* using pET28a plasmids successfully. The study provided scientific data for inquiring into the hereditary traits of the gene from Giant Panda and formulating the protective strategy for the Giant Panda.

2. Materials and methods

Materials and RNA isolation

Skeletal muscle was collected from a dead giant panda at the Wolong Conservation Center of the Giant Panda, Sichuan, China. The collected skeletal muscle was frozen in liquid nitrogen and then used for RNA isolation.

Total RNAs were isolated from about 400mg of muscle tissue using the Total Tissue/Cell RNA Extraction Kits (Waton Inc., Shanghai, China) according to the manufacturer's instructions. The total RNAs extracted were dissolved in DEPC (diethypyrocarbonate) water, and kept at-70℃.

Primers Design, RT-PCR, Cloning of RT-PCR Products and Sequencing

The PCR primers were designed by Primer Premier 5.0, basing on the mRNA sequence of alpha-B from *Homo sapiens* (NM_001885), *Mus musculus* (NM_009964), *Rattus norvegicus* (NM_012935). The specific primers of alpha-B are as follows:

alpha-B-F: 5′-GCCGCCATGGACATCGCCA -3′;

alpha-B-R: 5′-CTACTTCTTGGGGGCTGCAG-3′

Total RNAs were synthesized into the first-stranded cDNAs using a reverse transcription kit with Oligo dT as the primers according to the manufacturer's instructions (Promega). The 20μL of first-strand cDNA synthesis reaction system included 1μg of total RNAs, 5mM of $MgCl_2$, 1mM of dNTPs, 0.5μg of Oligo dT_{15}, 10U/μL of RNase inhibitor, and 15U of AMV reverse transcriptase, and was incubated at 42℃ for 60 minutes.

The first-strand cDNA synthesized was used as a template. The total reaction volume for DNA amplification was 25μL. Reaction mixtures contained 1.5mM of $MgCl_2$, 200μM of each of dATP, dGTP, dCTP and dTTP (Omega), 0.3μM of each primer, 5.0 units of Taq plus DNA polymerase (Sangon Co., Shanghai, China). DNA amplification was performed using a MJ Research thermocycler, Model PTC-200 (Watertown, MA) with a program of 4 minutes at 94.0℃, followed by 30 cycles of 1 minute at 94.0℃, 0.5 minute at 45℃ and 1.5 minutes at 72.0℃, and then ended with the final extension for 10 minutes at 72.0℃. After amplification, PCR products were separated by electrophoresis in 1.5% agarose gel with 1× TAE (Tris-acetate-EDTA) buffer, stained with ethidium bromide and visualized under UV light. The expected fragments of PCR products were harvested and purified from gel using a DNA harvesting kit (Omega, China), and then ligated into a pET28a vector at 22℃ for 12 hours. The recombinant molecules were transformed into *E. coli* complete cells (JM109), and then spread on the LB-plate containing 50μg/mL ampicillin, 200mg/mL IPTG (isopropyl-beta-D-thiogalactopyranoside), and 20mg/mL X-gal. Plasmid DNA was isolated and digested by *Pst*I and *Sca*II to verify the insert size. Plasmid DNA was sequenced by Huada Zhongsheng Scientific Corporation (Beijing, China).

Cloning the Genomic sequence of *CRYAB*

The PCR primers were the same as the alpha-B -F and alpha-B -R presented above. The genomic sequence of the *CRYAB* gene was amplified using Touchdown-PCR with the following conditions: 94℃ for 30s, 62℃ for 45s, 72℃ for 4 minutes in the first cycle and the anneal temperature deceased 0.5℃ per cycle; after 20 cycles conditions changed to 94℃ for 30s, 52℃ for 45s, 72℃ for 4 minutes for another 20 cycles. The fragment amplified was also purified, ligated into the clone vector and tansformed into the *E. coli* competent cells. Finally, the recombinant fragment was sequenced by Sangon (Shanghai, China).

Construction of the Expression Vector and Overexpression of Recombinant CRYAB

PCR fragment corresponding to the CRYAB polypeptide was amplified from the *CRYAB* cDNA clone with the forward primer, 5′- TCG AAT TCA TGG ACA TCG CCA -3′ (EcoRI) and reverse primer, 5′- CTA AGC TTG CGT CTA CTT CTT -3′ (HindIII), respectively. The PCR was performed at 94℃ for 3 minutes; 35 cycles of 30s at 94℃, 45s at 53℃ and 1 minutes at 72℃;10 minutes at 72℃. The amplified PCR product was cut and ligated into corresponding site of pET28a vector (Stratagen). The resulting construct was transformed into *E.coli* BL21(DE3) strain (Novagen) and used for the induction by adding IPTG (isopropyl-b-D-thiogalactopyranoside) at an OD600 of 0.6 and culturing further for 4 hours at 37℃, using the empty vector transformed BL21(DE3) as a control. The recombinant protein samples were induced after 1, 2

and 3 hours, and then separated by SDS-PAGE and stained with Commassie blue R 250.

SDS-PAGE analysis

The culture was centrifuged at 10000 g for 5 min at room temperature after it was induced for 0.5,1,2,3,4 hours, respectively. The culture supernatant was concentrated with methanol and chloroform (3:1,v/v),and SDS–PAGE(SDS polyacrylamide gel electrophoresis)was performed to investigate protein production and purity using slab gels containing 12%(w/v) polyacrylamide on a mini-protean II slab cell apparatus (Bio-Rad,Hercules,CA). Protein samples were visualized by Coomassie brilliant blue R-250 staining. The samples from the SDS–PAGE gels were transferred to a membrane of polyvinylidene difluoride (PVDF, Millipore, Billerica, MA), as previously described [28].

Data analysis

The sequence data were analyzed by GenScan software (http://genes.mit.edu/GENSCAN.html). Homology research of the Giant Panda *CRYAB* compared with the gene sequences of other species ware performed using Blast 2.1 (http://www.ncbi.nlm.nih.gov/blast/). ORF of the DNA sequence was searched using ORF finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein structure of the *CRYAB* sequence cloned was deduced using PredictProtein software (http://cubic.bioc.columbia.edu/predictprotein/).

3. Results

Analysis of the cDNA of *CRYAB* from the Giant Panda

About 530bp of cDNA fragment was amplified from the Giant Panda with the primers *CRYAB* -F and *CRYAB* -R (Fig. 1). The length of the cDNA cloned is 534bp. Blast research showed that the cDNA sequence cloned is highly homologous with the *CRYAB* from *Homo sapiens* and some other mammals reported. On the basis of the high identity, we concluded that the cDNA isolated is the cDNA encoding the Giant Panda CRYAB protein. The *CRYAB* sequence has been submitted to Genbank (accession number: FJ169483). An ORF of 528bp encoding 175 amino acids was found in the cDNA sequence (Fig. 2).

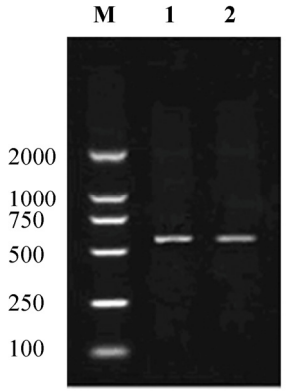

Fig. 1 Reverse Transcription Polymerase Chain Reaction Products of the Giant Panda *CRYAB*. M: Molecular ladder DL2000; 1, 2: the amplified *CRYAB*.

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1     GCCGCCATG GAC ATC GCC ATC CAC CAC CCT TGG ATC CGC CGC CCC TTC TTC CCC TTC
1            M   D   I   A   I   H   H   P   W   I   R   R   P   F   F   P   F
58   CAT TCT CCC AGC CGC CTC TTT GAC CAG TTC TTC GGG GAG CAC CTG TTG GAG TCT GAC
18    H   S   P   S   R   L   F   D   Q   F   F   G   E   H   L   L   E   S   D
115   CTC TTC CCA ACT TCT ACT TCC CTG AGC CCC TTC TAC CTG CGG CCA CCC TCA TTC CTG
37    L   F   P   T   S   T   S   L   S   P   F   Y   L   R   P   P   S   F   L
172  CGG GCA CCC AGC TGG ATT GAC ACC GGG CTC TCG GAG ATG CGT CTG GAG AAG GAC AGA
56    R   A   P   S   W   I   D   T   G   L   S   E   M   R   L   E   K   D   R
229  TTC TCT GTC AAC CTG GAT GTG AAG CAC TTC TCC CCA GAG GAA CTC AAG GTC AAG GTG
75    F   S   V   N   L   D   V   K   H   F   S   P   E   E   L   K   V   K   V
286  TTG GGA GAT GTG ATT GAG GTG CAC GGC AAA CAT GAA GAG CGC CAG GAT GAA CAT GGT
94    L   G   D   V   I   E   V   H   G   K   H   E   E   R   Q   D   E   H   G
343  TTC ATC TCC CGG GAG TTC CAC AGG AAA TAC CGG ATC CCA GCC GAT GTG GAC CCT CTT
113   F   I   S   R   E   F   H   R   K   Y   R   I   P   A   D   V   D   P   L
400  GCC ATT ACT TCA TCC CTG TCA TCT GAT GGG GTC CTC ACT GTG AAT GGA CCA AGG AAG
132   A   I   T   S   S   L   S   S   D   G   V   L   T   V   N   G   P   R   K
457  CAG GCC TCT GGC CCT GAG CGC ACC ATT CCC ATC ACC CGT GAA GAG AAG CCT GCT GTC
151   Q   A   S   G   P   E   R   T   I   P   I   T   R   E   E   K   P   A   V
514  ACT GCA GCC CCC AAG AAG TAG
170   T   A   A   P   K   K   *
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Fig. 2 Nucleotide and deduced amino acid sequences of cDNA enoding the Giant Panda *CRYAB*.

Analysis of the genomic sequence of *CRYAB* from the Giant Panda

A fragment of 3189 bp was amplified from genonic DNA of the Giant Panda using primers alpha-B -F and alpha-B –R. Comparison between the cDNA sequence and this DNA fragment indicated that the cDNA sequence is a full cDNA corresponding to three exons in the the *CRYAB* genomic sequence of the Giant Panda. The genomic sequence of the *CRYAB* gene has been submitted to Genbank (accession number: FJ439506).

Prediction and analysis of protein functional sites in CRYAB protein of the Giant Panda

Primary structure analysis revealed that the molecular weight of the putative CRYAB protein of the Giant Panda is 20.0668kDa with a theoretical pI 6.76 (Table 2). Topology prediction shows there are only four Casein kinase II phosphorylation sites in the CRYAB protein of the Giant Panda (Fig. 3).

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rat-p   MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFSTATSLSPFYLRPPSFLRAPSWID  62
hom-p   MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFPTSTSLSPFYLRPPSFLRAPSWFD  62
bos-p   MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFPASTSLSPFYLRPPSFLRAPSWID  62
mus-p   MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFSTATSLSPFYLRPPSFLRAPSWID  62
pd-p    MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFPTSTSLSPFYLRPPSFLRAPSWID  62

rat-p   TGLSEMRMEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHR  120
hom-p   TGLSEMRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHR  120
bos-p   TGLSEMRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHR  120
mus-p   TGLSEMRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHR  120
pd-p    TGLSEMRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHR  120

rat-p   KYRIPADVDPLTITSSLSSDGVLTVNGPRKQASGPERTIPITREEKPAVTAAPKK  175
hom-p   KYRIPADVDPLTITSSLSSDGVLTVNGPRKQVSGPERTIPITREEKPAVTAAPKK  175
bos-p   KYRIPADVDPLAITSSLSSDGVLTVNGPRKQASGPERTIPITREEKPAVTAAPKK  175
mus-p   KYRIPADVDPLTITSSLSSDGVLTVNGPRKQVSGPERTIPITREEKPAVAAAPKK  175
pd-p    KYRIPADVDPLAITSSLSSDGVLTVNGPRKQASGPERTIPITREEKPAVTAAPKK  175
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Fig. 3 Functional Sites in CRYAB Among the Giant Panda, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Bos Taurus.* rat: *Rattus norvegicus*; bos: *Bos Taurus*; homo: *Homo sapiens*; mus: *Mus musculus*; pd: the Giant panda; ▬: Casein kinase II phosphorylation site, ▒: polymorphic sites.

Overexpression of the *CRYAB* gene in *E. coli*

The *CRYAB* gene was overexpressed in *E. coli*, using pET28a plasmid, which carries a strong promoter and terminator sequences derived from phage T7. For this purpose, the C*RYAB* gene was amplified individually by PCR and cloned into the pET28a plasmid, resulting in a gene fusion coding for a protein bearing a GST-tag extension at the N terminus. Expression was tested by SDS-PAGE analysis of protein extracts from recombinant in *E. coli* BL21 strains (Fig. 4). The results indicated that the protein CRYAB fusion with the N-terminally His-tagged form gave rise to the accumulation of an expected 24kDa polypeptide that formed inclusion bodies. Apparently, the recombinant protein was expressed after half an hour of induction and the after 3 hours reached the highest level. These results suggested that the protein is active and it is just the protein encoded by the *CRYAB* from the Giant Panda. The expression product obtained could be used to purify the protein and study its function further.

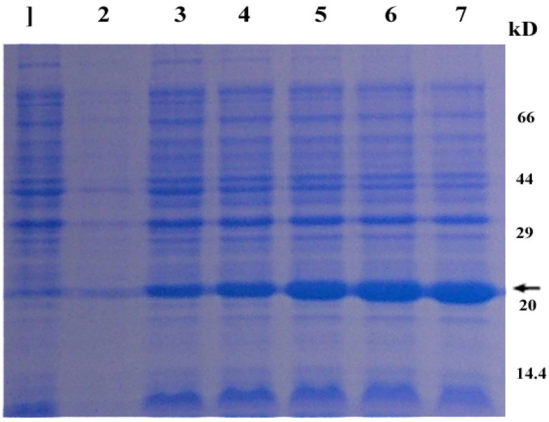

Fig. 4 Protein extracted from recombinant *E. coli* strains were analyzed by SDS-PAGE gel stained with Commassie blue R 250. Numbers on right shows the molecular weight, and the arrow indicates the recombinant protein bands induced by IPTG with 0, 0.5, 1, 2 ,3 and 4 hours (lane 2-7), respectively. The lane 1 represents the products of the *E. coli* strains with the empty vectors.

4. Discussions

Alignment analysis of *CRYAB* among the Giant Panda and those of *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Bos taurus*, indicated that both the nucleotide sequence and the deduced amino acid sequence are highly conserved. There is not any deletion and insertion of nucleotide and amino acid residue. Among them, the Giant Panda shares the highest homology for nucleotide sequence from *Bos taurus*; and the same highest homology for amino acid sequences (Table 1). Physical and chemical analysis showed that the molecular weight of the putative protein among the five mammalians is very close and that the theoretical pI is exactly identical (Table 2).

Table 1 Comparison of Nucleotide and Amino Acid Sequences among 5 Mammal Species.

	A. melanoleuca	*H.sapiens*	*M. musculus*	*R. norvegicus*	*B. taurus*
A. melanoleuca		93.9%	91.5%	91.5%	95.3%
H. sapiens	98.3%		88.5	88.4%	91.1%
M. musculus	97.1%	97.7%		93.1%	86.5%
R. norvegicus	97.7%	97.1%	98.3%		87.1%
B. taurus	99.4%	97.7%	96.6%	97.1%	

Note: The homology matrix of *CRYAB* encoding sequence is above the diagonal, the homology matrix of protein sequence is below the diagonal.

Table 2 Molecular Weight and pI of CRYAB of the Giant Panda and Other Four Mammals.

	A. melanoleuca	*H. sapiens*	*M. musculus*	*R. norvegicus*	*B. taurus*
Molecular weight(kDa)	20.0668	20.1589	20.0688	20.0888	20.0368
pI	6.76	6.76	6.76	6.76	6.76

The genomic sequence of *CRYAB* is 3189 bp in size, containing three exons and two introns. Compared with some mammals incluing *Homo sapiens* (NC_000011), *Mus musculus* (NC_000075), *Rattus norvegicus* (NC_005107) and *Bos taurus* (NC_007313), the length of the genomic, the first intron, the second intron, the 5′-untranslated sequence and the 3′-untranslated sequence are different (see Table 3).

Table 3 Comparison of *CRYAB* genomic among 5 Mammal Species.

	Length of genomic	Number of exons	Number of introns	Length of 5′-untranslated sequence	Length of 3′-untranslated sequence	Length of first intron	Length of second intron
Ailuropoda melanoleuca	3189	3	2	6	0	1113	1542
Homo sapiens	3124	3	2	25	138	1074	1359
Mus musculus	3876	3	2	519	148	1034	1667
Rattus norvegicus	4253	3	2	579	137	1029	1970
Bos taurus	3226	3	2	0	81	1127	1480

It is known that αB-crystallins undergo post-translational modifications, including truncation of both the N terminus and the C terminus, deamidation, racemization, phosphorylation, methionine oxidation, glycation, disulfide formation, addition of O-GlcNAc, and the addition of 72 mass units to the C-terminal lysine of B-crystallin [29-34]. Some of these activities, such as phosphorylation and specific cleavage, may be functionally important; others are likely the result of aging and detrimental stresses. aB-crystallin has polar, flexible C-terminal extensions that is thought to contribute to the solubility of this crystallin and that has been implicated in the chaperone-like activity of this crystallin [35]. Topology prediction revealed there are four Casein kinase II phosphorylation sites in the CRYAB proteins of the five mammalian species. Our analysis indicate that the same number and patter functional sites are located in the same locations in CRYAB protein of the Giant Panda, *Homo sapiens*, *Rattus norvegicus*, *Mus musculus* and *Bos Taurus* (Fig. 3) and the amino acid sequence deduced from Giant Panda shares the highest homology with that of *Bos Taurus*. Further analysis detected eight polymorphic sites in the amino acid sequences of the five species compared. These polymorphic sites are located irregularly in the amino acid sequences all of which result from the transversion or transition of the corre-

sponding codons without any deletion and insertion of base. Among these polymorphic sites, only site 61 is located in Casein kinase II phosphorylation site, but it does not result in any differences from human and other four mammalian species in the functional site. The fact shows that the variation of sites has no affect on the structure and function of CRYAB protein. However, what changes caused by other mutations outside the functional sites in the structure and the function of CRYAB need further studies.

In addition, it was reported that in the interactive β3 sequence, $_{73}$DRFSVNLDVKHFS$_{85}$ of human αB crystallin, Ser-76, Asn-78, Lys-82, and His-83 were identified as nonconserved residues on the exposed surface of the a crystallin core domain. Chaperone activity was influenced by the amount of unfolding of the target proteins and independent of complex size. It was reported that the importance of the exposed side chains of Glu-78, Lys-82, and His-83 in the interactive β3 sequence of the α crystallin core domain in aB crystallin for chaperone function. The β3 strand was conserved at 5 of 8 positions, and 4 of the 5 conserved residues were buried inside the β sandwich structure of the α crystallin core domain where the side chains were positioned to form intramolecular bonds. In contrast, the side chains of the nonconserved residues were surface exposed, suggesting their involvement in protein–protein interactions with homologous sHSP subunits and/or unfolded chaperone target proteins at the surface of the a crystallin core domain [36,37]. It was reported that that amino acid differences at positions 76, 78, 82, and 83 in the β3 sequence could account for the difference in chaperone activities. On the basis of numbers of research findings above, we speculate that the most important sequence of our predicted *CRYAB* protein of the Giant Panda for its structure and function is the sequence: $_{73}$DRFSVNLDVKHFS$_{85}$. A comparison of the β3 sequence of Giant Panda aB crystalline with those of *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Bos Taurus* shows that amino acid share the same homology and the position Ser-85 is located in the first site of a Casein kinase II phosphorylation site (Fig. 3). It indicates that the β3 sequence in αB crystallin may be an interactive site that selects target proteins for chaperone activity on the basis of the degree of unfolding and may be a chaperone site that is selective for partially and completely unfolded proteins.

In Summary, the cDNA and the complete coding sequence of *CRYAB* gene has been cloned and the *CRYAB* cDNA is expressed efficiently in prokaryotic organism using pET28a plasmids. The gained fusion protein is in accordance with the expected 24kDa polypeptide. These results suggest that the protein is active and it is just the protein encoded by the *CRYAB* from the Giant Panda. Further study on CRYAB protein will contribute to the genetic polymorphism and the protection for gene resources of Giant Panda.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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