• Basic Research •

Recombination and identification of human alpha B-crystallin

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Abstract

• AIM: To recombine the human alpha B-crystallin (αB-crystallin) using gene cloning technology and prokaryotic expression vector and confirm the biological activity of recombinant human αB-crystallin.

• METHODS: Cloning the human αB-crystallin cDNA according to the nucleotide sequence of the human αB-crystallin, constructing the pET-28/CRYAB prokaryotic expression plasmid by restriction enzyme digestion method, and stably expressing transformed into the Escherichia coli (E. coli) DH5 alpha. The recombinant human αB-crystallin was purified by Q sepharose. By enzyme digestion analysis, Western blotting and sequencing, the recombinant human αB-crystallin was identified and the activity of its molecular protein was detected.

• RESULTS: Compared with the gene bank (GeneBank), the cloned human sequence of human αB-crystallin cDNA has the same open reading frame. Identification and sequencing of the cloned human αB-crystallin cDNA in prokaryotic expression vector confirmed the full length sequence, and the vector was constructed successfully. The E. coli containing plasmid pET-28/CRYAB induced by isopropyl-β-D-thiogalactoside successfully expressed the human αB-crystallin. Insulin confirmed that the recombinant human αB-crystallin has a molecular chaperone activity.

• CONCLUSION: The prokaryotic expression vector pET-28/CRYAB of recombinant human αB-crystallin is successfully constructed, and the recombinant human αB-crystallin with molecular chaperone activity is obtained, which lay a foundation for the research and application of the recombinant human αB-crystallin and its chaperone activity.

• KEYWORDS: human alpha B-crystallin; gene; vector construction; identification; recombination

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INTRODUCTION

Alpha B-crystallin (αB-crystallin) is a subunit of alpha crystallin. In 2002, αB-crystallin was first isolated from the lens by Srivastava[1]. Later studies have shown that αB-crystallin is one of a small molecular heat shock protein (sHsps) family, which is sHspB5. αB-crystallin is 20 kD and has the commonness of sHsps family, including small molecular weight, participation in the process of cell development, and being able to resist stress responses and apoptosis induced by various factors[2]. The recent research demonstrated that αB-crystallin is related to age-related macular degeneration, optic neuropathy due to glaucoma, diabetic retinopathy, retinal tear, retinal ischemia-reperfusion injury, retinal dystrophy, and other pathological changes[3-9].

The abilities of αB-crystallin inhibiting protein aggregation, oxidation, inflammation, and apoptosis have been exploited for its therapeutic use in eye diseases. Exogenous administration of αB-crystallin inhibits microglial activation and rescues optic nerve oligodendrocytes in an experimental animal model of anterior ischemic optic neuropathy in mice[10]. In addition to the whole protein, a recent study showed that the administration of αB-crystallin derivatives have also shown promising results. The intraperitoneal injection of αB-crystallin derivatives inhibited drug-induced cataracts in rats, which was accompanied by their beneficial effects against protein aggregation and lens epithelial cell apoptosis[11].

The therapeutic uses of αB-crystallin for other diseases outside the eye have also been reported. Following central nervous system injury in mice, the administration of human αB-crystallin led to an improvement in locomotor skills and an

inhibition of secondary tissue damage during the acute stage[12]. In many kinds of animal models, such as cerebral ischemia, autoimmune encephalomyelitis, multiple sclerosis, diabetes, autoimmune myocarditis, and cardiac ischemia, the exogenous administrations of αB-crystallin or its hexameric peptide showed significant therapeutic benefits[13-19].

However, complete αB-crystallin is unable to be separated from alpha crystallin, which limits further research and application. This study used an Escherichia coli (E. coli) prokaryotic expression system to recombine human αB-crystallin, as well as to study the protective effect of the recombinant αB-crystallin, and lay a foundation for its clinical application in treating optic nerve injury.

MATERIALS AND METHODS

Experimental Materials

Plasmids, strains and cells E. coli BL21 (DE3) (Novagen company, USA); PMD19-T vector (Takara company, Japan); E.coli DH5α strain (Novagen company, USA); pET28a (Novagen company, USA).

The main enzymes and reagents Reverse transcription Kit (Toyobo company, Japan); DNA gel extraction kit (Tiangen company, Germany); plasmid DNA extraction kit (Tiangen company, Germany); Luria Bertani medium (Difco, USA); KOD plus DNA polymerase (Toyobo company, Japan); restriction endonucleases Nco I, hind I and EcoRI and xhoi (Toyobo company, Japan); DNA ligase solution I (Takara company, Japan); polymerase chain reaction (PCR) reagents Taq polymerase (Japan Takara company, Japan).

Methods

Obtain human alpha B-crystal protein gene The sequences of human αB-crystallin was retrieved from the GenBank: mdiaihhpwi rrpffpfhsp srlfdqffge hllesdlfpt stslspfylr ppsflrapsw fdtglsemrl ekdrfsvnld vkhfspeelk vkvlgdviev hgkheerqde hgfisrefhr kyripadvdp ltitsslssd gvltvngprk qvsgpertip itreekpavt aapkk. According to the codon usage bias of E. coli, the amino acid sequence of human αB-crystallin was transformed into DNA sequence.

Polymerase chain reaction amplification of target gene According to the gene sequence of human αB-crystallin and the multiple cloning sites of the vector, the specific primer of human αB-crystallin gene was designed by Primer 5 software. Upstream primer: 5'-GGAATTGATCGCCATCCACCAC-3' and downstream primer: 5'-CCGCTCGAGCTATTTCTT GGGGGCTGCGG-3'. The primers were synthesized by the biotechnology Service Co., Ltd of Shanghai, China, and the length of the amplified fragment was 545 bp. After PCR amplification, the amplified products were analyzed by electrophoresis in 1 g/L agarose gel, and the results were analyzed by gel auto imaging system.

Construction and identification of target gene vector plasmid Recovery and purification of target gene from PCR products after agarose gel electrophoresis (referring the specification of Tiangen company general agarose gel DNA Extraction Kit), purified DNA fragment of 50 μL (0.96 ng/μL), dNTP 5 uL (2 mmol/L) and Taq enzyme 1 μL (5 μ/μL), at 72℃, 20min, Poly A tail is added to the 3' end of the DNA. The synthesized gene fragment was then inserted into the PMD19-T vector at restriction enzyme sites EcoRI and XhoI. The products were transformed into E. coli BL21 (DE3) pLysS. A single colony of positive PMD19-T-αB-crystallin. E.coli BL21 (DE3) pLysS was inoculated into 5 mL LB medium containing ampicillin and incubated overnight at 37℃, in a 150-rpm shaker. The target gene PCR was identified by taking the bacterial fluid, and the recombinant vector was sent to the sequencing company for sequencing. At the same time, the recombinant plasmid of PMD19-T-αB-crystallin was extracted and identified by EcoR I and Xho I double enzyme digestion.

Inducible expression of recombinant proteins pET28a-αB-crystallin plasmid was transformed into E. coli BL21 (DE3) pLysS. A single colony of positive pET28a-αB-crystallin. E. coli BL21 (DE3) pLysS was inoculated into LB medium and incubated overnight at 37℃ in a 150-rpm shaker. The overnight culture was inoculated into fresh LB medium and incubated at 37℃, at 150 rpm until the culture reached an optical density at 600 nm of 0.6-0.8. This was followed by addition of isopropyl-β-D-thiogalactoside (IPTG) solution to induce protein expression (in a 150-rpm shaker, 30℃, overnight), and a number of final concentrations of IPTG (0, 0.2, 0.4, 0.6 and 0.8 mmol/L) of recombinant protein expression induction were evaluated. The cells were collected after recombinant protein expression was terminated. Then the harvested cells were resuspended in phosphate-buffered saline (PBS) and lysed. The lysate was centrifuged in a 150-rpm shaker, at 4℃, for 15min. The supernatant was analyzed using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of recombinant Trx-αB-crystallin fusion protein The recombinant protein contains Thioredoxin and His Tags, which was purified by Ni2+/IDA metal chelating affinity column chromatography followed by Q-Sepharose ion-exchange column to obtain high purity fusion protein. The purity of the target protein was evaluated using SDS-PAGE.

Identification of recombinant human αB-crystallin and detection of molecular chaperone activity The recombinant human αB-crystallin was identified by PAGE, Western-blot and protein peptide mass fingerprinting. Insulin reduction assay was used to detect the chaperone activity of the recombinant human αB-crystallin *in vitro*, which can inhibit the aggregation of insulin B chain caused by reducing agent and suppressing sediment formation[20].

RESULTS

The Amplification of the Target Gene Fragment The synthesized target DNA fragment was ligated into the PMD19-T vector in order to construct the recombinant plasmid PMD19-T-αB-crystallin, which was then digested by restriction endonuclease Ecor l and XhoI. Gel electrophoresis revealed that the cleavage of Ecor l and XhoI generated the target fragment, which was consistent with the expected fragment size of 545 bp (Figure 1A). This indicates that the target gene fragment was successfully inserted into the PMD19-T vector.

Expression and Purification of the Recombinant Alpha B-crystallin

Construction of the expression vector The synthesized target DNA fragment was ligated into the pET-28a vector in order to construct the expression vector pET-28a-αB-crystallin, which was then digested by restriction endonuclease Ecor l and XhoI. Gel electrophoresis revealed that the cleavage of Ecor l and XhoI generated the target fragment, which was consistent with the expected fragment size of 545 bp (Figure 1B). The endonuclease cleavage fragment was verified by sequencing, and the results of this were consistent with the αB-crystallin sequence, indicating successful construction of the expression vector.

Expression of the recombinant αB-crystallin The pET-28a-αB-crystallin recombinant plasmid was transformed into E. coli BL21 (DE3) pLysS, protein expression was induced using 0.4 and 0.6 mmol/L IPTG under varying conditions. Upon terminating the expression, 10% SDS-PAGE was used to verify differing protein expression (Figure 2). The target protein was expressed in the pellet, which showed the recombinant αB-crystallin expression was successfully induced in E. coli BL21 (DE3) pLysS.

Purification of the recombinant αB-crystallin Ni2+/IDA metal chelating affinity column chromatography and Q-Sepharose ion-exchange column were conducted to purify the target protein (Figure 3). SDS-PAGE revealed that the size of the purified target protein matched its predicted size of 20 kDa, with above 95% purity (Figure 4).

Identification of Recombinant Proteins

Coomassie Brilliant Blue staining of the recombinant αB-crystallin The recombinant human αB-crystallin during PAGE was stained with Coomassie Brilliant Blue. A recombinant human αB-crystallin band with the size of about 20 kD can be observed (Figure 5A). The size of the recombinant human αB-crystallin was similar to that found in GenBank, suggesting that the size of the recombinant protein was correct.

Western-blot analysis of the recombinant αB-crystallin Anti-αB-crystallin monoclonal antibody was used in Western blot analysis to assess the expressed recombinant αB-crystallin. The recombinant protein was demonstrated to specifically bind the anti-αB-crystallin monoclonal antibody, which showed

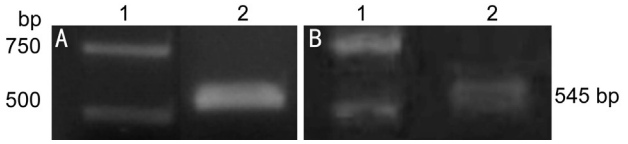

Figure 1 Gel electrophoresis following Ecor l and XhoI double enzymatic digestion A: Recombinant plasmid PMD19-T-αB-crystallin; B: Recombinant plasmid pET28a-αB-crystallin. Lane 1: Marker; Lane 2: Target gene fragment.

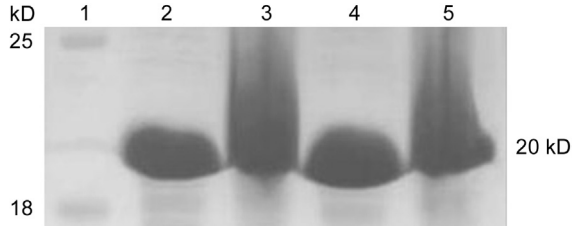

Figure 2 Recombinant protein expression SDS-PAGE Lane 1: Marker; Lane 2-5: 0.4 mmol/L and 0.6 mmol/L IPTG (supernatant and pellet).

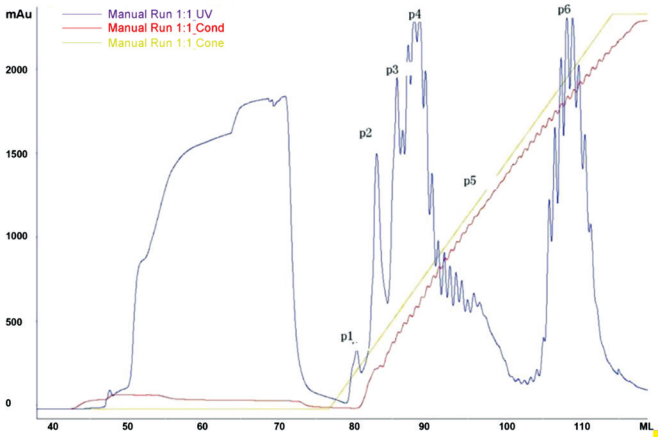

Figure 3 Purification of the recombinant αB-crystallin by Q-Sepharose ion-exchange column.

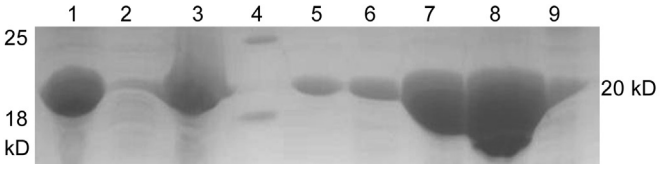

Figure 4 Recombinant protein purified by chromatography Lane 1-3: Simples, supernatant, pellet; Lane 4: Marker; Lane 5-9: p1, p2, p3, p4, p5 purified protein.

the recombinant αB-crystallin were successfully constructed (Figure 5B).

Peptide mass fingerprinting analysis of the recombinant αB-crystallin Searching in MATRIX SCIENCE database and using Mowse Score, when the score is greater than 83, the peptide fragments of the identified peptides match with the known protein peptide fragments in the database, there was significant difference (P<0.05; Figure 6). The peptide mass fingerprinting showed that the recombinant protein bands is αB-crystallin (Figure 7).

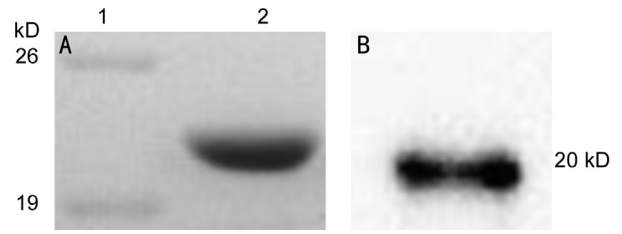

Figure 5 Coomassie Brilliant Blue staining (A) and Western blot analysis (B) of the recombinant protein Lane 1: Marker; Lane 2: Recombinant human αB-crystallin.

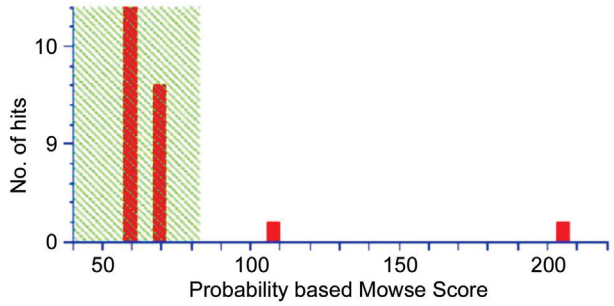

Figure 6 Peptide mass fingerprinting analysis of the recombinant protein.

gi|4503057 Mass: 20146 Score: 205 Expect: 3.5e-014 Queries matched: 21
alpha-crystallin B chain [Homo sapiens]
gi|30584657 Mass: 20260 Score: 205 Expect: 3.5e-014 Queries matched: 21
Homo sapiens crystallin, alpha B [synthetic construct]
gi|2852648 Mass: 22435 Score: 180 Expect: 1.1e-011 Queries matched: 20
unknown [Homo sapiens]
gi|227018373 Mass: 20127 Score: 158 Expect: 1.7e-009 Queries matched: 17
alpha B crystallin [Homo sapiens]
gi|117386 Mass: 20062 Score: 156 Expect: 2.7e-009 Queries matched: 17
RecName: Full=Alpha-crystallin B chain; AltName: Full=Alpha(B)-crystallin
gi|16905067 Mass: 20076 Score: 156 Expect: 2.7e-009 Queries matched: 17

Figure 7 Identification of the recombinant protein by peptide mass fingerprint.

Identification of molecular chaperone activity of the recombinant αB-crystallin The molecular chaperone activity of the recombinant human αB-crystallin was determined by insulin reduction assay, and compared with the negative control group (Figure 8). The results showed that the recombinant human αB-crystallin inhibited the aggregation and precipitation of insulin in the presence of reducing agent DTT. The experimental results show that the recombinant human αB-crystallin has molecular chaperone activity (Figure 9).

DISCUSSION

Human αB-crystallin is an sHsps; the molecular weight is small, about 20 kD and 170 to 180 amino acids[21]. αB-crystallin is a widely expressed sHsp first identified in the lens, and has been implicated in the pathogenesis of many diseases, including neurodegenerative disorders, myopathies, cancer, and cataracts[22-30]. The molecular chaperone activity of αB-crystallin is the core of its biological function, which plays an important part in the activity of life. In ophthalmology, the molecular chaperone activity of αB-crystallin protects the optic nerve and prevents the lens from ultraviolet radiation injury[31]. Because human αB-crystallin cannot be separated from the alpha crystallin, limiting its research and application.

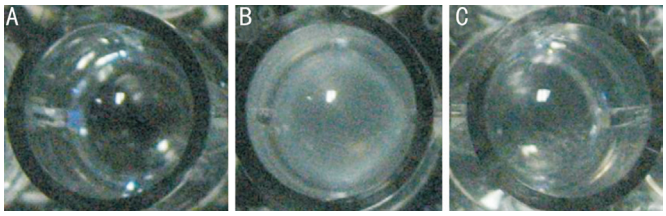

Figure 8 Insulin reduction assay A: Negative group (Deionized water); B: Positive group (Insulin+DTT); C: Recombinant human αB-crystallin group (Insulin+DTT+αB-crystallin).

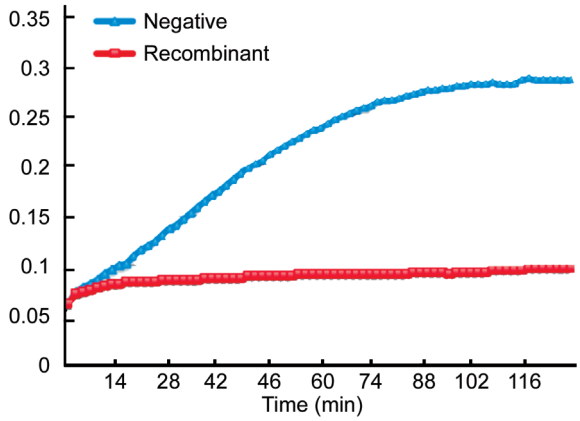

Figure 9 Recombinant human αB-crystallin molecular chaperone activity test.

Recombinant αB-crystallin with molecular chaperone activity is the basis for its further application. In this study, the gene engineering method was used to clone the human αB-crystallin gene fragment into the expression vector, the recombinant protein was then expressed and purified.

At present, the E. coli expression system is the most mature; it is also the first and most widely used of the recombinant protein expression systems. E. coli expression system offers several advantages such as high expression level, stability, and simple operation. Therefore, a recombinant human B-crystallin was prepared in this study using the E. coli prokaryotic expression system, and the biological activity of the recombinant protein was evaluated.

pET system is a widely used expression vector system for recombinant protein production. pET vector makes the cloning, expression, and purification of recombinant proteins easier[32-34]. A pET vector system using T7RNA polymerase and promoter matching system allows the cloning and expression to successfully leave. Many of the genes that are difficult to be expressed can efficiently and stably be cloned and expressed by the PET system.

On the basis of the above experiments, the double enzyme digestion method was used to insert the target protein gene into the pET28a plasmid vector. The vector pET-28a also has a T7 promoter, as well as exhibiting the advantages of low induced leakage, simplicity, convenience, rapid induction, and efficient expression of multiple genes. The biggest advantage for this study is that the pET28a plasmid vector can completely

express the target protein. Further, the expressed recombinant protein has no label and can be purified without a restriction enzyme, which not only simplifies the experimental procedure, but also protects the recombinant protein activity. In this study, identified pET28a-αB-crystallin recombinant plasmid was transformed into E. coli. After inducing expression, a significant recombinant protein molecular mass appeared at 20 kD band. This was revealed by protein electrophoresis and Western blot analysis, which is consistent with the target protein αB-crystal protein. The expression level of the recombinant protein is high, and the target protein was more than 30% of the total protein. The researches above indicated that the prokaryotic expression system of recombinant human αB-crystallin was successfully constructed. The recombinant protein was then purified by Q column chromatography and microfilter, and the purity of which reached over 95%. The recombinant protein was identified by peptide mass fingerprint mass spectrometry, which indicated that the target protein was human αB-crystallin. In conclusion, the prokaryotic expression system of pET28a is a stable and efficient method to prepare recombinant human αB-crystallin.

αB-crystallin (sHspB5) is one of the ten well-known members of sHsps belonging to mammalian heat shock proteins; it has the general characteristics of small molecule heat shock protein-molecular chaperone activity. In 1978 Laskey first proposed the concept of molecular chaperone, which is also known as molecular chaperone. Ellis and van der Vies[35] extended it to a class of proteins with molecular chaperone activity that is widely distributed in the body, its function is to mediate the accurate folding and assembly of proteins, and to protect the protein activity, but itself is not a functional component of the final assembly. Hendrick and Hartl[36] proposed that the molecular chaperone is a class of proteins that combine with other proteins in an unstable conformation and make them stable. Molecular chaperone by controlling the binding and separation help it's binding protein to fold, assembly, transport and degradation *in vivo*[36-37]. In 1992, Horwitz[38] confirmed that the alpha crystallin had molecular chaperone function, and which inhibited the thermal aggregation of b and g-crystalline protein, maintaining the transparency of the lens. Moreover, the molecular chaperone function of alpha crystallin has an inhibitory effect on the nonspecific agglutination reaction when the lens is exposed to UV irradiation, chemical modification, embellishment, and other kinds of denaturing agents, which is essential to maintain the transparency of the lens[39]. The alpha crystalline has two subunits, alpha A and alpha B, both of which have molecular chaperone activity. However, the molecular chaperone activity of αB-crystallin is 3 times of alpha A-crystallin in human normal temperature condition. In this study, we confirmed that the recombinant human αB-crystallin has molecular chaperone activity by the classical insulin chaperone activity test[40].

In summary, this study successfully constructed the prokaryotic expression vector carrying exogenous human αB-crystallin gene, obtained recombinant human αB-crystallin through expression and purification, and proved that the recombinant human αB-crystallin has distinct molecular chaperone activity *in vitro*. The conclusion of this study has established a solid foundation for further applications of the recombinant human αB-crystallin in the treatment of optic nerve injury.

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Conflicts of Interest: Wang R, None; **Chen ZH,** None; **Wang Y,** None; **Huang HB,** None; **Fan SJ,** None; **Chen LL,** None.

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