BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **244,** 131–137 (1998)
ARTICLE NO. RC988226

Characterization, Cloning, and Expression of Porcine αB Crystallin

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Received January 20, 1998

α-Crystallin is a major lens protein present in the lenses of all vertebrate species. Recent studies have revealed that bovine α-crystallins possess genuine chaperone activity similar to small heat-shock proteins. In order to compare this chaperone-like structural protein from the eye lenses of different mammalian species, we have cloned and expressed one of the main α-crystallin subunits, i.e., αB crystallin, from the porcine lenses in order to facilitate the structure-function evaluation and comparison of this chaperonin protein. cDNA encoding αB subunit chain was obtained using a new "Marathon cDNA amplification" protocol of Polymerase Chain Reaction (PCR). PCR-amplified product corresponding to αB subunit was then ligated into *p*GEM-T plasmid and prepared for nucleotide sequencing by the dideoxynucleotide chain-termination method. Sequencing several positive clones containing DNA inserts coding for αB-crystallin subunit constructed only one complete full-length reading frame of 525 base pairs similar to human and bovine αB subunits, covering a deduced protein sequence of 175 amino acids including the universal translation-initiating methionine. The porcine αB crystallin shows only 3 and 7 residues difference to bovine and human αB crystallins respectively, revealing the close relatedness among mammalian eye lens proteins. The sequence differences between porcine and submammalian species such as chicken and bullfrog are much greater, especially at the N- and C-terminal regions of these αB crystallins. Expression of αB subunit chain in *E. coli* vector generated a polypeptide which can cross-react with the antiserum against the native and purified αB subunit from the native porcine lenses albeit with a much lower activity. © 1998 Academic Press

α-Crystallin is one of the best studied proteins with respect to posttranslational modifications, including age-induced alterations due to its long life in the eye lens (1). The α-crystallins of most vertebrate lenses consist of αA and αB subunit chains showing about 55-60% sequence similarity. They were previously regarded as lens-specific proteins and of exclusively structural nature (2). Ingolia and Craig (3) first reported that heat shock proteins of *Drosophila* showed sequence similarity to mammalian α-crystallin. Recently they have been found to be expressed in a variety of extra-lenticular tissues suggesting that there may be a general cellular function for these proteins (4–6). This protein is believed to play an important role in the maintenance of lens transparency (1). α-Crystallin shows structural and functional similarities to small heat-shock proteins (7); similarly its expression can also be induced by thermal (8) and osmotic stress (9). *In vitro* studies of α-crystallin also indicate chaperone-like activity of this lens protein in preventing the aggregation of other proteins (10,11). However, the detailed molecular mechanism for its chaperone-like action remains unknown.

In this report, a major interest has been focused on the cloning and characterization of porcine αB crystallin, which is not well studied both at the protein and gene levels when compared with that obtained from bovine lenses. We have amplified cDNAs constructed from the lenses of pigs employing a newer "Marathon cDNA amplification" protocol of PCR methodology to aid in the structural analysis of αB crystallin subunit chains. cDNA clone encoding the αB-crystallin chain of this mammalian species has been sequenced successfully, thus circumventing the need for time-consuming process of protein sequencing due to the presence of a blocking group at the amino-terminus (1 and the references therein). It is also deemed essential to determine αB-crystallin sequence for interspecies structure/function comparison in light of the recent interesting find-

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ings that various α-crystallins possess genuine chaperone activity similar to that of heat-shock proteins (10,12). Comparison of the chaperone activity of αA and αB crystallin subunits isolated from native lenses and expressed αB crystallin product indicates that in contrast to the previous reports, αB from both native or recombinant source possesses less activity than αA after *in vitro* refolding, which may reflect their structural difference in the primary structure resulting in different crystallin aggregates responsible for chaperone activity.

MATERIALS AND METHODS

Isolation and characterization of frog α-crystallin. The pig (*Sus scrofa* var. *domestica*) lenses used were obtained from a local slaughterhouse. The isolation and purification of porcine α-crystallin was essentially as described before (13,14). The α-crystallin fraction from gel filtration column was further purified into its αA and αB subunits on reversed-phase HPLC according to the previous report (15).

Preparation of mRNA from porcine lenses and cloning by PCR amplification. Lens total poly(A)$^+$ RNA of porcine lenses was purified using the QuickPrep mRNA preparation kit (Pharmacia, Uppsala, Sweden). Double-strand cDNA synthesis was carried out with the Marathon cDNA amplification kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) according to the manufacturer's instructions. The PCR amplification was carried out using either a forward or reverse primer corresponding to the ligated adaptor in combination with an αB-specific forward primer, 5′-ATGGACATCGCCATCCACCAC-3′ (3′-RACE) or an αB-specific reverse primer, 5′-CTTCTCTTCACGGGTGATGGG-3′ (5′-RACE). The final amplification using a primer at the 5′ end, *i.e.* 5′-C(A/T)(C/T)ACA(C/T)TCACCTAGCCACCATG-3′ within the 5′-noncoding region (with the initiator codon underlined), and a primer with the stop codon at 3′-noncoding region, *i.e.* 5′-ATGCAGTTAGTGGAAGGGCATCTA-3′ (with stop codon underlined) were used to amplify the complete coding region from double-strand cDNA. The PCR products were purified on 1.2% agarose gel. The purified products were ligated into *p*GEM-T (Promega, Madison, WI, USA) for sequence analysis by automatic fluorescence-based sequencing using a model 373A DNA Sequencing System with a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc., CA, U.S.A.).

Expression and purification of αB-crystallin. The forward primer, *i.e.* 5′-GCCATATGGACATCGCCATCCACCACC-3′) corresponding to the 5′-end, contains an ATG initiator codon (CATATG) and an *Nde*I restriction site. The reverse primer (5′-GCAAGCTTCTACTTCTTGGGGGCTGCAG-3′) was complementarily overlapped with the 3′-end of the coding region containing the *Hind*III site (AAGCTT) linked to the translational stop codon. A nucleotide fragment for the porcine αB-crystallin was synthesized by PCR amplification of the porcine αB-crystallin open-reading frame of the plasmid. The PCR product was purified, double-digested with *Nde*I and *Hind*III and then ligated into *Nde*I/*Hind*III sites of the isopropyl β-D-thiogalactoside (IPTG)-inducible *E. coli* expression vector *p*ET21a(+) (Novogen Inc., U.S.A.). The resulting plasmid, *p*ET/ αB, was transformed into *E. coli* strain BL21(DE3) (Novogen Inc.). Transformants were selected on LB-agar plates supplemented with 100 μg/ml ampicillin. For induction of gene expression, *E. coli* BL21 cells containing recombinant plasmid were grown at 37°C in 1 liter of LB medium containing 100 μg/ml ampicillin. When the A_{600} of the growing culture reached 0.7, IPTG was added to a final concentration of 1 mM. Four hours after induction, cells were harvested and resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl) and lysed by ultrasonication. After ultrasonication, the mixture was then centrifuged for 20 min at 20,000 × g to remove any remaining insoluble debris. Soluble recombinant proteins were purified by gel filtration on Sepharose CL-6B, DEAE-Sephacel and followed by Sephacryl S-300HR. The purity of the recombinant αB-crystallin was confirmed by SDS-PAGE and reversed-phase HPLC.

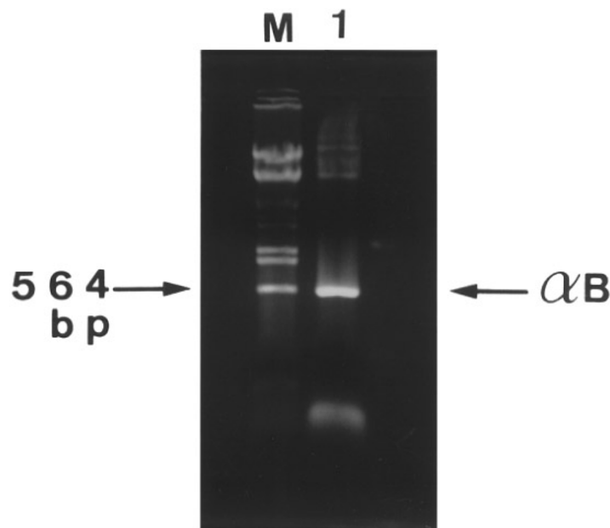

FIG. 1. Identification and size determination of PCR-amplified porcine αB-crystallin cDNA. Electrophoresis was carried out in 1.2% agarose gel. Lane M, DNA size markers of the *Eco*RI and *Hin*dIII digestion products of λDNA, ranging from 564 to 21227 bp; Lane 1 indicates the amplified PCR product of about 560 bp (arrow) encoding porcine αB-crystallin.

Gel electrophoresis and immunoblotting. SDS-polyacrylamide slab gel (5% stacking/ 14% resolving gel) electrophoresis (SDS-PAGE) was as described (16). For immunoblotting detection of αB-crystallin, the gels were subjected to electroblotting to a nitrocellulose membrane after SDS-PAGE followed by immunological analysis using mouse antiserum against porcine αB-crystallin and reacted with peroxidase-conjugated AffiniPure goat anti-mouse IgG+IgM(H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). A color development reaction was carried out using diaminobenzidine and hydrogen peroxide.

Amino acid and sequence analyses. Amino acid analysis and N-terminal sequence analysis by automated Edman degradation were carried out as described before (17).

Assay for chaperone activity of α-crystallin under thermal heating. Porcine βL-crystallin (195 μg) each was incubated with 14 μg α-crystallin, reversed-phase HPLC separated αA, αB and 6 M urea-treated αA, αB respectively. Six samples were all in phosphate-buffered saline (PBS) in a final volume of 1.5 ml. Thermal aggregation experiments were carried out at 60°C and the relative scattering was measured at 340 nm as described in the previous report (18). Porcine βL-crystallin (195 μg) in PBS heated alone was used as control. Concentrations of proteins were determined by a protein assay (Bio-Rad).

RESULTS AND DISCUSSION

α-Crystallins consisting of αA and αB subunit chains are major water-soluble proteins of the transparent eye lens, which have previously been regarded as lens-specific proteins (2). Recently they were found to be expressed in a variety of extra-lenticular tissues suggesting a general cellular function for these proteins (1

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                10          20          30          40          50          60
TCA CAC ATT CAC CTA GCC ACC ATG GAC ATT GCC ATC CAC CAC CCC TGG ATC CGC CGC CCC
                             M   D   I   A   I   H   H   P   W   I   R   R   P
                                                                   10

                70          80          90         100         110         120
TTC TTT CCT TTC CAC TCT CCC AGC CGC CTC TTT GAC CAG TTC TTT GGA GAA CAC CTG TTG
 F   F   P   F   H   S   P   S   R   L   F   D   Q   F   F   G   E   H   L   L
                         20                                      30

               130         140         150         160         170         180
GAG TCT GAT CTC TTC CCA GCT TCT ACT TCC CTG AGC CCC TTC TAC TTT CGG CCG CCC TCG
 E   S   D   L   F   P   A   S   T   S   L   S   P   F   Y   F   R   P   P   S
                         40                                      50

               190         200         210         220         230         240
TTC CTG CGG GCA CCC AGC TGG ATT GAC ACT GGG CTC TCA GAG ATG CGT CTG GAG AAA GAC
 F   L   R   A   P   S   W   I   D   T   G   L   S   E   M   R   L   E   K   D
                         60                                      70

               250         260         270         280         290         300
AGA TTC TCT GTC AAC CTG GAT GTG AAG CAC TTC TCC CCC GAG GAA CTC AAG GTC AAG GTG
 R   F   S   V   N   L   D   V   K   H   F   S   P   E   E   L   K   V   K   V
                         80                                      90

               310         320         330         340         350         360
TTG GGA GAT GTG ATT GAG GTG CAC GGC AAA CAT GAA GAG CGC CAG GAT GAA CAT GGT TTC
 L   G   D   V   I   E   V   H   G   K   H   E   E   R   Q   D   E   H   G   F
                        100                                     110

               370         380         390         400         410         420
ATC TCC CGG GAG TTC CAC AGG AAA TAC CGG ATC CCA GCT GAT GTG GAC CCT CTC ACC ATT
 I   S   R   E   F   H   R   K   Y   R   I   P   A   D   V   D   P   L   T   I
                        120                                     130

               430         440         450         460         470         480
ACT TCA TCC CTG TCA TCT GAT GGG GTC CTC ACT GTG AAT GGA CCA AGG AGA CAG GCC TCT
 T   S   S   L   S   S   D   G   V   L   T   V   N   G   P   R   R   Q   A   S
                        140                                     150

               490         500         510         520         530         540
GGC CCC GAG CGC ACC ATT CCC ATC ACC CGT GAA GAG AAG CCT GCT GTC ACT GCA GCC CCC
 G   P   E   R   T   I   P   I   T   R   E   E   K   P   A   V   T   A   A   P
                        160                                     170

               550         560         570
AAG AAG TAG ATG CCC TTC CAC TAA CTG CAT
 K   K   *
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FIG. 2. Nucleotide and deduced amino acid sequences of the porcine αB-crystallin subunit. The nucleotide sequence of 570 base pairs including the designed primer sequences is shown above the amino-acid sequence of 175 residues, which includes the translation initiation methionine as the first amino acid. The asterisk (*) indicates the stop codon TAG. cDNA sequence is marked in every 10-nucleotide segment for easy tracing of sequence contents. Amino acids are denoted by one-letter symbols. The underlines point to the 5′- and 3′-end primers outside the coding region used in the amplification of cDNA encoding αB-crystallin chain. The first initiation Met residue is *N*-terminally acetylated for all αA and αB chains after protein synthesis *in vivo.*

and references therein). There are currently more than 60 determined sequences of αA crystallin chains from varied species of vertebrates by conventional protein sequencing (19,20). However there have been fewer αB chains reported in the literature by conventional protein sequencing. In order to provide a means of correlating structure/function properties of α-crystallin with chaperone activity, it is deemed essential to complement the crystallin sequence information by the facile protocols of PCR methodology.

Isolation and Characterization of Porcine αB-Crystallin

Lens crystallins were similarly isolated by gel filtration as described previously (14). The α-crystallin fraction from gel filtration column was purified into its αA and αB subunits on C4-reverse phase HPLC column (15). Purified αA and αB subunits were treated with the denaturing buffer (6 M urea, 50 mM Tris/HCl, pH 8.0) and then dialyzed against refolding buffer (50 mM

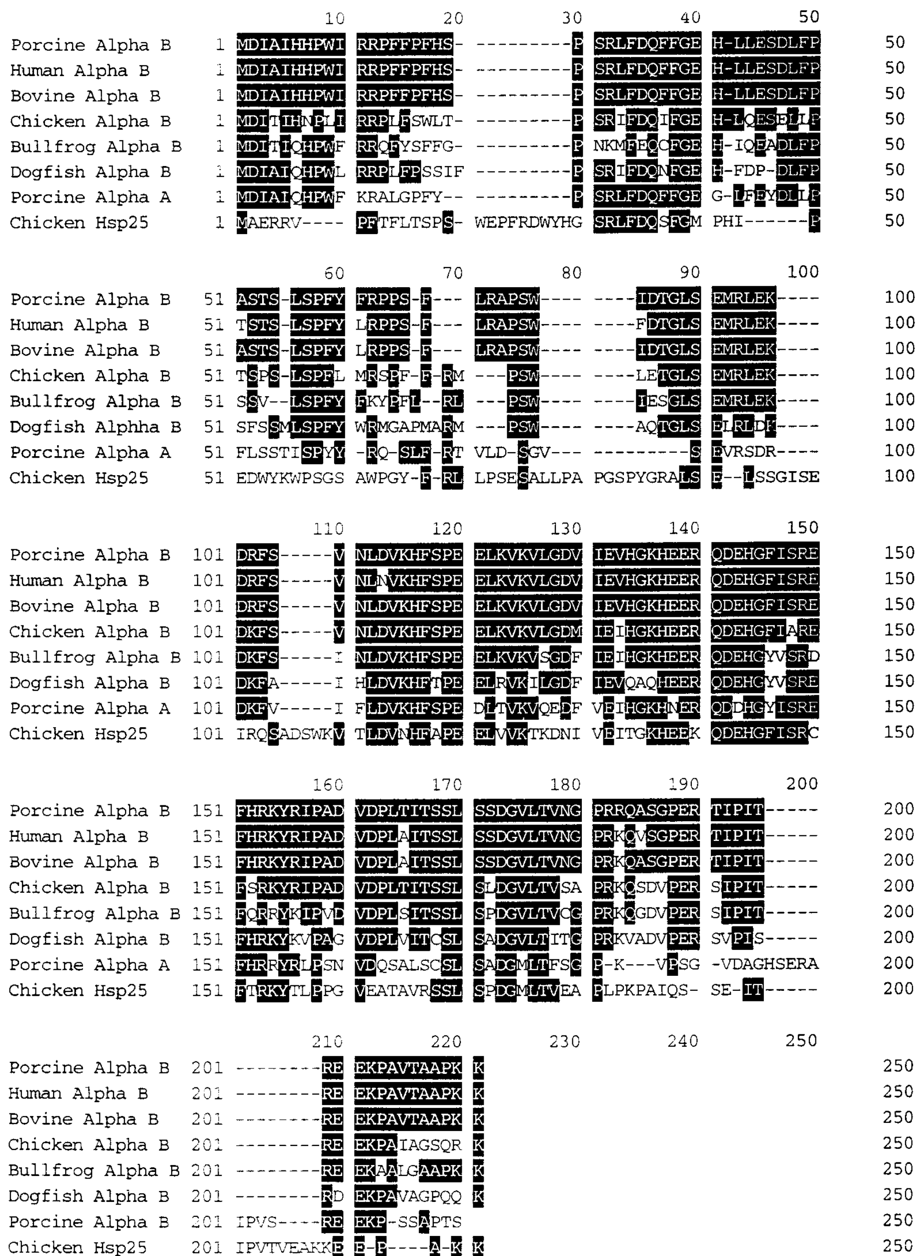

FIG. 3. Multiple sequence alignment and sequence comparison of seven α-crystallin subunits and chicken heat-shock protein (hsp25). The identical amino-acid residues among various sequences are expressed as white letters on black blocks. The gaps are introduced for optimal alignment and maximum homology among the sequences. The sequences listed are cited as follows: human αB (24), bovine αB (25), chicken αB (26), bullfrog αB (23), dogfish αB (27), porcine αA (28), and chicken hsp25 (29). Note that the N-terminal segments (residues 1-100) are more variable among these compared sequences whereas C-terminal sequences are more conserved.

Tris/HCl, pH 8.0). Amino acid analysis and N-terminal sequence analysis by automated Edman degradation were used to check the purities and sequences of subunit chains.

Cloning and Sequence Analysis by the "Marathon" Protocol of PCR cDNA Amplification

PCR amplification of total lens cDNA mixtures prepared from two lenses of a single pig with the designed primers based on the highly conserved mammalian αB-crystallin amino-acid sequence stretches PITREEK for 5′-RACE and MDIAIHH for 3′-RACE (21,22). We have also used two primers within the 5′ and 3′ noncoding region with either initiator codon or stop codon to achieve the amplification of a 0.56 kb cDNA fragment (**Fig. 1**), a size corresponding to a polypeptide of about 170-180 amino-acid residues. After checking for nucleotide sequences by automatic DNA sequencing, a cDNA

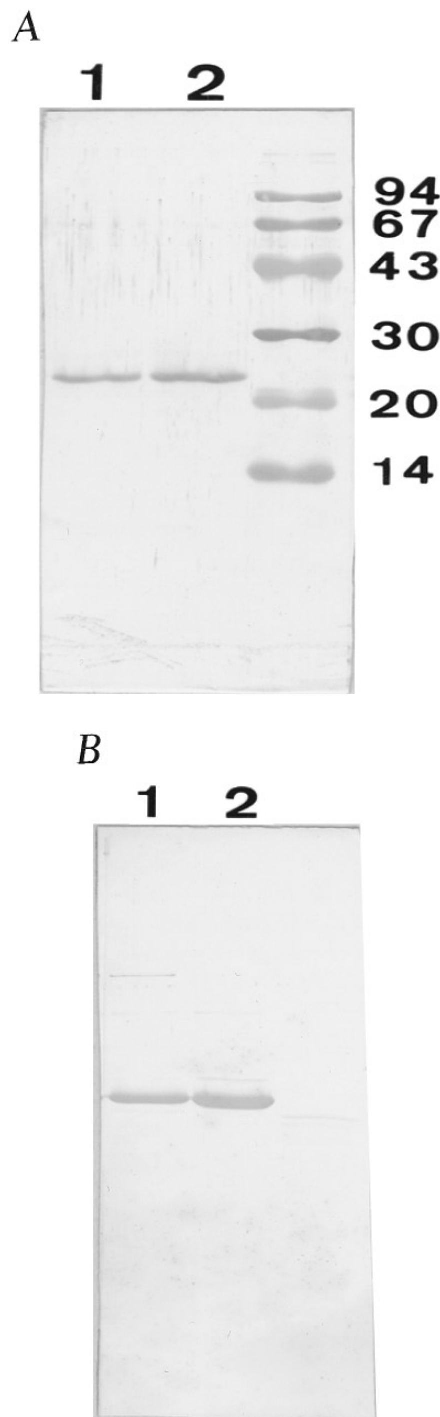

FIG. 4. Expression and immunoblotting analysis of porcine αB-crystallin. The gel was subjected to electroblotting onto a nitrocellulose membrane after SDS-PAGE followed by immunological analysis using mouse antiserum against porcine αB-crystallin. Lanes 1 and 2 represent expressed porcine αB-crystallin and purified native porcine αB-crystallin respectively. (A) Amido-black stained membranes, standard protein markers (in kDa) are shown on the right lane: phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20), and lysozyme (14); (B) Immunoblot stained with horseradish peroxidase conjugated goat anti-mouse IgG+IgM on the SDS-PAGE gel.

sequence covering an open reading frame of 564-nucleotides with the expected 5′ and 3′ primer sequences was obtained. The deduced amino acid sequence together with its nucleotide coding sequence is shown in **Fig. 2.** It is to be noted that we have sequenced several positive clones containing DNA inserts coding for pig αB-crystallin subunit, all sequencing data pointing to only one complete full-length reading frame of 525 base pairs similar to human and bovine αB subunits, covering a deduced protein sequence of 175 amino acids including the universal translation-initiating methionine. The use of "Marathon" PCR protocol for the amplification of cDNA is actually a combination of 5′- and 3′-Race PCR with the advantage of avoiding using primers covering the N- or C-terminal protein coding regions which may lead to some uncertainty or ambiguity in the 5′ and 3′ nucleotide coding regions after cDNA sequencing.

Sequence Alignment and Comparison of αB-Crystallins from Different Species

Fig. 3 shows the optimal alignment by introducing a minimum number of gaps along the entire lengths of eight determined sequences encompassing representative αB-crystallins from species of the major classes in vertebrates by multiple sequence alignment program. In the comparative analysis of sequence alignment, it is found that porcine αB crystallins show about 96-98% sequence homology to human and bovine αB crystallins. The porcine αB crystallin shows only 3 and 7 residues difference to bovine and human αB crystallins respectively, revealing the close relatedness among mammalian eye lens proteins. The sequence differences between porcine and submammalian species such as chicken (73% sequence homology) and bullfrog (68% sequence homology) are much greater, especially at the N- and C-terminal regions of these αB crystallins. It is noteworthy that there is only about 59% sequence homology between porcine αB crystallins and a more distantly related dogfish αB crystallin. The sequence homology between porcine αA and αB crystallins is about 49% which is similar to that of bovine αA and αB (23), underlining the distinct differences found between these two subunits of α-crystallin as judged by the sequence variation of these two polypeptide chains. One salient feature for the comparison between mammalian αB and chicken heat-shock protein is that there is only about 35-38% sequence similarity, well below the homology found among αB crystallins of various vertebrate species. In spite of lower sequence homology found between these two types of proteins, α-crystallin has been shown to possess chaperone activity similar to small heat-shock protein (10).

Expression and Immunological Comparison of Cloned and Native Porcine αB Crystallins

Since there are so far several reports on the successful expression of recombinant α-crystallins from bovine

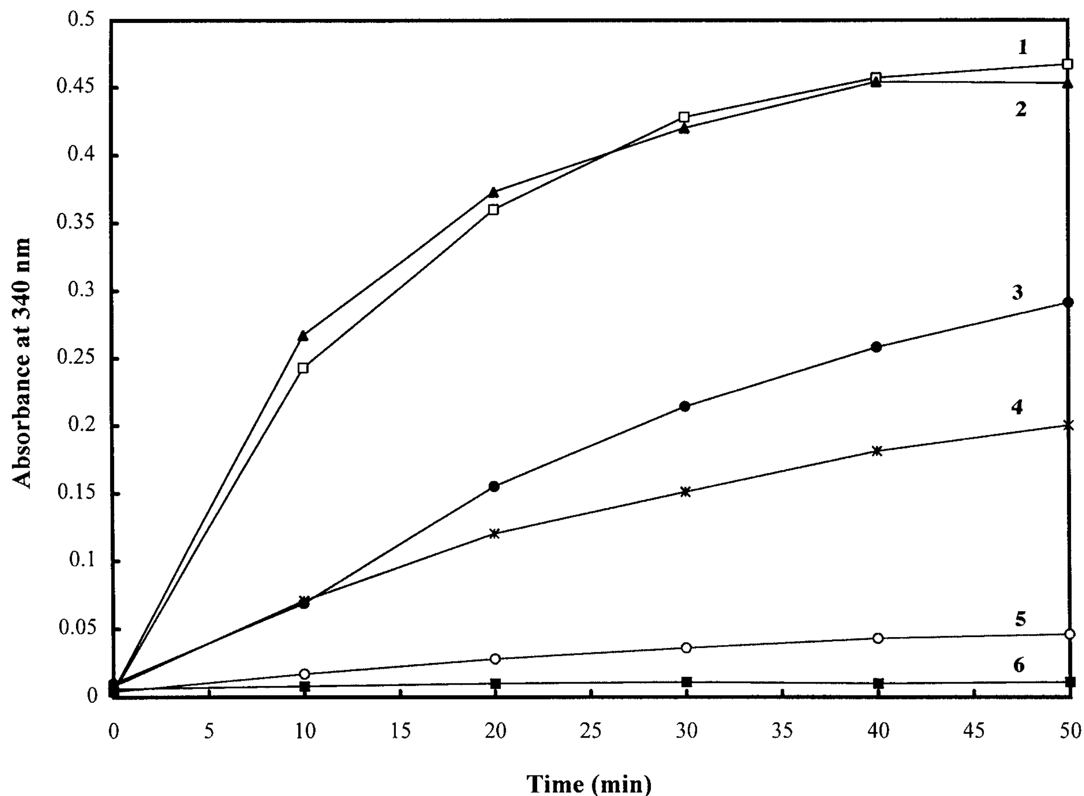

FIG. 5. Thermal aggregation of βL-crystallin at 60°C in the presence of α-, αA- or αB-crystallins. The concentration of βL-crystallin used in these experiments was 0.13 mg/ml whereas that of various α-crystallins was 0.0093 mg/ml. Concentrations of crystallin solutions used in the experiments were estimated by a protein assay (Bio-Rad). Curve 1, βL-crystallin control solution in the absence of α-crystallin; Curve 2, βL-crystallin in the presence of refolded recombinant αB-crystallin; Curve 3, βL-crystallin in the presence of 6 M urea-treated and refolded HPLC-purified αA-crystallin; Curve 4, βL-crystallin in the presence of 6 M urea-treated and refolded HPLC-purified αB-crystallin; Curve 5, βL-crystallin in the presence of HPLC-purified αA-crystallin without urea denaturation; Curve 6, βL-crystallin in the presence of native α-crystallin isolated from gel filtration.

or human sources in *E. coli* system, we have also adopted a similar expression approach by cloning the PCR-amplified fragment encoding αB-crystallin subunit in an *E. coli* expression vector. A nucleotide fragment for the porcine αB-crystallin was synthesized by PCR amplification of the porcine αB-crystallin open-reading frame of the plasmid. The PCR product was purified, double-digested with *Nde*I and *Hind*III and then ligated into *Nde*I/*Hind*III sites of the isopropyl β-D-thiogalactoside (IPTG)-inducible *E. coli* expression vector *p*ET21a(+). The resulting plasmid, *p*ET/αB, was transformed into *E. coli* strain BL21(DE3). For induction of gene expression, *E. coli* BL21 cells containing recombinant plasmid were grown at 37°C. Soluble recombinant proteins were purified by gel filtration on Sepharose CL-6B, DEAE-Sephacel and followed by Sephacryl S-300HR. The purity of the recombinant αB-crystallin was confirmed by SDS-PAGE and large-pore (C4) reversed-phase HPLC. To verify that the 22 kDa band on SDS-PAGE indeed corresponded to porcine αB-crystallin, the recombinant protein purified by reversed-phase HPLC was also subjected to Edman sequence determination for 15 cycles. The sequencing result revealed that N-terminal sequence of this protein (MDIAIHHPWIRRPFF) was identical to the deduced amino-acid sequence predicted from the αB cDNA sequence. Further confirmation of the expressed porcine αB-crystallin product was demonstrated by the Western immunoblotting analysis using antiserum against purified porcine αB-crystallin isolated from pig lenses (**Fig. 4**). It is noteworthy that antiserum against native αB-crystallin did cross-react specifically only with expressed products and no reactions were detected with control lysate without IPTG induction.

Comparison of Chaperone Activity of Native and Recombinant αA and αB Crystallins

In the present study we have used porcine βL-crystallin as the substrate for thermal heating experiments to measure and compare the chaperone-like activity of α-crystallins from native and recombinant sources. As shown in **Fig. 5,** the rapid aggregation of βL-crystallin in the absence of α-crystallin during incubation at 60°C

is evident (curve 1) whereas the turbidity of βL-crystallin solution is almost completely inhibited in the presence of native α-crystallin (about 0.01 mg/ml, curve 6). We have also compared the chaperone activity for αA, αB directly obtained from reversed-phase HPLC and refolded αA, αB after 6 M urea denaturation together with recombinant αB after purification and refolding. It is noteworthy that the recombinant αB (curve 2) and native and urea-treated refolded αB (curve 4) all show less activity than native α (curve 6) and αA without urea denaturation (curve 5). The reasons for this discrepancy are probably due to the formation of improper folding αB aggregates after expression or there may be some undetected contaminant proteins from *E. coli* expression system which may interfere with refolding or inhibit the chaperone activity *per se.* We are currently refining the purification and refolding protocols for the recombinant αB crystallin in *E. coli* expression system with the aim of achieving the correct folding aggregate with higher chaperone activity for structure-function study of αB crystallin.

CONCLUSION

Insights into the mechanism of chaperone-like activity associated with α-crystallin and its subunit αA and αB chains may be revealed through the analysis and comparison of molecular sequences and structures from eye lenses of different animal species. In this study we have established the nucleotide sequence of cDNA clone encoding the major αB crystallin subunit from porcine eye lenses by a facile Marathon-Race protocol of PCR methodology. In light of the recent intense interest in studies on the structural and functional similarity between α-crystallin and heat shock proteins and their associated chaperone activity, characterization of αA/αB crystallin from more diverse species both at the structural and functional levels may provide important insights on the molecular origin and underlying mechanism for the evolution of chaperone activity associated with this major class of crystallins in the animal kingdom.

ACKNOWLEDGMENTS

This work was supported by Academia Sinica and the National Science Council (NSC Grants 83-0203-B-001-086, 83-0418-B-001-020BA, 84-2311-B-001-050-BA & 86-2311-B-002-031-B15), Taipei, Taiwan.

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