

Comparative analysis and molecular characterization of genomic sequences and proteins of *FABP4* and *FABP5* from the giant panda (*Ailuropoda melanoleuca*)

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ABSTRACT. Fatty acid binding proteins (FABPs) are a family of small, highly conserved cytoplasmic proteins that bind long-chain fatty acids and other hydrophobic ligands. In this study, cDNA and genomic sequences of *FABP4* and *FABP5* were cloned successfully from the giant panda (*Ailuropoda melanoleuca*) using reverse transcription polymerase chain reaction (RT-PCR) technology and touchdown-PCR. The cDNAs of *FABP4* and *FABP5* cloned from the giant panda were 400 and 413 bp in length, containing an open reading frame of 399 and 408 bp, encoding 132 and 135 amino acids, respectively. The genomic sequences of *FABP4* and *FABP5* were 3976 and 3962 bp, respectively, which each contained four exons and three introns. Sequence alignment indicated a high degree of homology

with reported FABP sequences of other mammals at both the amino acid and DNA levels. Topology prediction revealed seven protein kinase C phosphorylation sites, two casein kinase II phosphorylation sites, two N-myristoylation sites, and one cytosolic fatty acid-binding protein signature in the *FABP4* protein, and three N-glycosylation sites, three protein kinase C phosphorylation sites, one casein kinase II phosphorylation site, one N-myristoylation site, one amidation site, and one cytosolic fatty acid-binding protein signature in the *FABP5* protein. The *FABP4* and *FABP5* genes were overexpressed in *Escherichia coli* BL21 and they produced the expected 16.8- and 17.0-kDa polypeptides. The results obtained in this study provide information for further in-depth research of this system, which has great value of both theoretical and practical significance.

Key words: Genomic sequence; Overexpression; *FABP4* and *FABP5*; Cloning; Giant panda; *Ailuropoda melanoleuca*

INTRODUCTION

Fatty acid-binding proteins (FABPs) are abundant intracellular proteins that bind long-chain fatty acids with high affinity. Nine separate mammalian FABPs have been identified to date, whose tertiary structures are highly conserved (Choromańska et al., 2011). FABPs have unique tissue-specific distributions that have long suggested functional differences among them. In the last decade, considerable progress has been made in understanding the specific functions of FABPs and, in some cases, their mechanisms of action at the molecular level (Storch and Thumser, 2010; Smathers and Petersen, 2011). FABPs appear to be involved in the extranuclear compartments of the cell by trafficking their ligands within the cytosol via interactions with organelle membranes and specific proteins (Storch and McDermott, 2009; Monaco, 2009). Several members of the FABP family have been shown to function directly in the regulation of cognate nuclear transcription factor activity via ligand-dependent translocation to the nucleus. It is thought that FABPs' roles include fatty acid uptake, transport, and metabolism (Uysal et al., 2000; Veerkamp and Zimmerman, 2001; Makowski and Hotamisligil, 2005; Maeda et al., 2005; Xu et al., 2007; Storch and Corsico, 2008). *FABP4* encodes the fatty acid-binding protein found in adipocytes, while *FABP5* encodes the fatty acid-binding protein found in epidermal cells (Boord et al., 2002; Makowski and Hotamisligil, 2004). *FABP4* predicts the development of the metabolic syndrome independently of pubertal status, adiposity, and insulin resistance (Grundy et al., 2005; Tuncman et al., 2006), whereas *FABP5*, first identified as being upregulated in psoriasis tissue, is associated with type 2 diabetes (Hotamisligil, 2005; Krusinová and Pelikánová, 2008; Bu et al., 2011), oral squamous cell carcinoma (Fang et al., 2010), and some neurological diseases, such as autism, schizophrenia (Maekawa et al., 2010), and bipolar disorder (Iwayama et al., 2010). For these reasons, FABPs have been recently suggested as a potential therapeutic target of these abnormalities in animal models (Gong et al., 2010; Choi et al., 2011).

The giant panda (*Ailuropoda melanoleuca*) is a rare species that is currently found only in China. It is at high risk of extinction due to human population expansion and conse-

quent destruction of its habitat. At present, there is very little genetic information about the panda (Du et al., 2007; Hou et al., 2008a,b; Zhang et al., 2009), which is an essential tool for obtaining a detailed understanding of the biology of this organism. Recently, the giant panda's draft genome sequence, which has good coverage and completeness for genes with unique sequences, was generated and assembled (Li et al., 2010); however, some genes are still incomplete in this project.

In this study, reverse transcription polymerase chain reaction (RT-PCR) was used to amplify the cDNA of *FABP4* and *FABP5* from the total RNAs, which were extracted from the skeleton muscle of the giant panda. The genomic sequences of *FABP4* and *FABP5* were cloned successfully from the giant panda using touchdown-PCR. We also analyzed the sequence characteristics of the protein encoded by the cDNA and compared it with those reported for humans and other mammalian species. This study provides information regarding structure and function of *FABP4* and *FABP5* and characteristics of the panda genome, and contributes a solid foundation for developing deeper molecular research, formulating a protective strategy, and making proper use of the panda's genetic resources.

MATERIAL AND METHODS

Material

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 DNA and RNA isolation.

RNA and DNA isolation

Total RNAs were isolated from approximately 400 mg muscle tissue using the Total Tissue/Cell RNA Extraction Kits (Waton Inc., Shanghai, China) according to manufacturer instructions, and was then dissolved in RNase-free ddH₂O and stored at -70°C. Genomic DNA was isolated from the muscle tissue of the giant panda, according to previously described methods (Hou et al., 2008a,b). The cDNA obtained was dissolved in TE buffer and kept at -20°C. DNA and RNA sample quality was checked using Experion (Bio-Rad, China) and quantification was performed spectrophotometrically.

Primer design, RT-PCR, cloning of cDNA sequences, and sequencing

Premier 5.0 was used to design primers. The primers of *FABP4* were designed based on mRNA sequences from *Homo sapiens* (BC003672), *Bos taurus* (X89244), *Ovis aries* (EU301804), and *Rattus norvegicus* (BC084721). The primers of *FABP5* were designed based on mRNA sequences from *H. sapiens* (BC019385), *B. taurus* (BT020981), *O. aries* (FJ546079), and *R. norvegicus* (S83247). The primer sequences were as follows: *FABP4*-F: 5'-TTCTCAC CTTGAAGAATAAT-3'; *FABP4*-R: 5'-GTTTCGATGAAAACCTTCAGCT-3'; *FABP5*-F: 5'-CCG CACCCACCATGGCCACC-3'; *FABP5*-R: 5'-AGTATGGAGATTTGCTCATT-3'.

First-stranded cDNAs were synthesized using a reverse transcription kit with oligo dT as the primers, followed by PCR amplification, according to manufacturer instructions (Pro-

mega, Shanghai, China). Reverse transcription reactions were performed in duplicate. Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples in the absence of cDNA synthesis. After amplification, PCR products were separated by electrophoresis on 1.5% agarose gel with 1X TAE buffer, stained with ethidium bromide, and visualized under ultraviolet light. The expected fragments of PCR products were harvested and purified from the gel using a DNA harvesting kit (Omega Bio-Tek, USA), and were then ligated into a pMD19-T vector (TaKaRa, Dalian, China) at 16°C for 2 h. The recombinant molecules were transformed into *Escherichia coli* competent cells (DH5 α) and then spread on the Luria-Bertani plate containing 50 μ g/mL ampicillin, 200 mg/mL isopropyl- β -D-1-thiogalactopyranoside (IPTG), and 20 mg/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 the Huada Zhongsheng Scientific Corporation (Beijing, China).

Cloning the genomic sequences of *FABP4* and *FABP5*

The genomic DNA of *FABP4* was amplified using primers *FABP4*-F and *FABP4*-R by touchdown-PCR with the following conditions: 94°C for 30 s, 48°C for 45 s, and 72°C for 2 min in the first cycle, and the annealing temperature decreased by 0.2°C per cycle; after 20 cycles, conditions changed to 94°C for 30 s, 52°C for 45 s, and 72°C for 2 min for another 20 cycles.

The genomic DNA of *FABP5* was amplified using primers *FABP5*-F and *FABP5*-R2 (5'-TGATGATGGA ATTTTACTC-3') also by touchdown-PCR with the same conditions described above, except that the initial annealing temperature was 50°C.

Next, the amplified fragment was also purified, ligated into the clone vector, and transformed into the *E. coli* competent cells. Finally, the recombinant fragment was sequenced by the Huada Zhongsheng Scientific Corporation.

Construction of the expression vector and overexpression of recombinant *FABP4* and *FABP5*

The PCR fragment corresponding to the *FABP4* polypeptide was amplified from the *FABP4* cDNA clone with the forward primer: 5'-GCGGATCCATGTGTGATGCCTTTG-3' (*Bam*HI) and the reverse primer: 5'-GCGAATTCTTATGCTCTCTCATAA-3' (*Eco*RI). PCR was performed at 94°C for 3 min; 30 cycles of 30 s at 94°C, 45 s at 52°C, 1 min at 72°C, and 10 min at 72°C.

The PCR fragment corresponding to the *FABP5* polypeptide was amplified from the *FABP5* cDNA clone with the forward primer: 5'-GCGGATTCATGGCCACCATTTCAGC-3' (*Bam*HI) and the reverse primer: 5'-GCGAATTCTTACTCTACTTTTCA-3' (*Eco*RI). PCR was performed under the same conditions as above except that the annealing temperature was 49°C.

The amplified PCR product was cut and ligated into the corresponding site of the pET28a vector (Stratagene, USA). The resulting construct was transformed into the *E. coli* BL21 (DE3) strain (Novagen, USA) and used for induction by adding IPTG at an OD₆₀₀ of 0.6, and culturing further for 4 h at 37°C using the empty vector transformed BL21 (DE3) as a control. The recombinant protein samples were induced after 0, 0.5, 1, 2, 2.5, 3, 3.5, and 4

h, and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue R 250.

Data analysis

The genomic DNA and cDNA sequences were analyzed using GenScan. The homology search was performed using Blast 2.1. Multiple sequence alignment was performed with DNAMAN6.0. Base bias and substitution was analyzed with MEGA 3.1. The open reading frame (ORF) of the DNA sequence was searched using the ORF Finder software. The protein structure of the cloned *FABP4* and *FABP5* sequences was deduced using the PredictProtein software. The advanced protein structure was analyzed using Jpred and SWISS-MODEL.

RESULTS

Analysis of the genomic sequences of *FABP4* and *FABP5* from the giant panda

DNA fragments of nearly 4000 bp were amplified with primers *FABP4-F*, *FABP4-R*, *FABP5-F*, and *FABP4-R*. The length of the DNA fragments cloned from the giant panda were 3976 and 3962 bp, which were both found to possess four exons and three introns. Comparisons between the cDNA sequence and the DNA fragment sequences of *FABP4* and *FABP5* amplified from the giant panda were performed using DNAMAN version 6.0. The result indicated that the cDNA sequences were in full accordance with four fragments in the DNA fragment, suggesting that these amplified DNA fragments were the genomic sequences of *FABP4* and *FABP5* from the giant panda. The genomic sequences of *FABP4* and *FABP5* of the giant panda have been submitted to GenBank (accession Nos. JN008913 and JN008914).

Compared with previous reports of some mammals, the four exons comprising the cDNA sequence of *FABP4* and *FABP5* after RNA splicing were found to be highly conserved, being essentially identical. The restriction sites in the exons are the same in both the cDNA and the genomic sequences. In contrast, the genomic, introns, 5'-untranslated, and 3'-untranslated sequences differed in length (see Tables 1 and 2). Variations in the lengths of the introns determine the lengths of the *FABP4* and *FABP5* genes.

Table 1. Comparison of genomic sequence of *FABP4* among 4 mammal species.

	<i>A. melanoleuca</i>	<i>H. sapiens</i>	<i>M. musculus</i>	<i>B. taurus</i>
Genome length (bp)	3975	4742	4208	4391
Exon number	4	4	4	4
Intron number	3	3	3	3
5'-Untranslated region length sequence (bp)	37	70	173	60
3'-Untranslated region length sequence (bp)	34		43	167
Join sites in the CDS	38..110, 2562..2734, 3320..3421, 3891..3941 JN008912	71..144, 2641..2813, 3724..3825, 4324..4724 NC_000008.10	174..223, 894..996, 1603..1775, 4092..4165 NC_000069.5	61..133, 2843..3015, 3610..3711, 4175..4225 NC_007312.4

Table 2. Comparison of genomic sequence of *FABP5* among 5 mammal species.

	<i>A. melanoleuca</i>	<i>H. sapiens</i>	<i>M. musculus</i>	<i>B. taurus</i>	<i>S. scrofa</i>
Genome length (bp)	3962	4224	4005	6777	4755
Exon number	4	4	4	4	4
Intron number	3	3	3	3	3
5'-Untranslated region length sequence (bp)	11	46	34	50	192
3'-Untranslated length sequence (bp)	14	206	490	1364	82
Join sites in the CDS	12..90, 2684..2856, 3229..3330, 3895..3948	47..123, 2815..2986, 3321..3421, 3964..4018	35..111, 2364..2536, 2865..2967, 3461..3515	51..127, 4045..4216, 4576..4679, 5359..5413	193..243, 1453..1557, 1936..2107, 4594..4673
GenBank accession Nos.	JN008913	NC_000008.10	NC_000069.5	NC_007312.4	NC_010446.2

Analysis of the cDNA of *FABP4* and *FABP5* from the giant panda

The cDNA fragments of approximately 400 bp were amplified from the giant panda with the primers. The cDNA sequences of *FABP4* and *FABP5* were 400 and 413 bp, respectively. The BLAST search showed that the cloned cDNA sequences shared a high degree of homology with those of mammalian species reported in the NCBI database. On the basis of the high level of sequence identity, we concluded that we had cloned cDNAs encoding the giant panda *FABP4* and *FABP5* proteins. The cDNA sequences have been submitted to GenBank (accession Nos. JN008911 and JN008912). Their ORFs are 399 and 408 bp, encoding 132 and 135 amino acids, respectively (Figure 1).

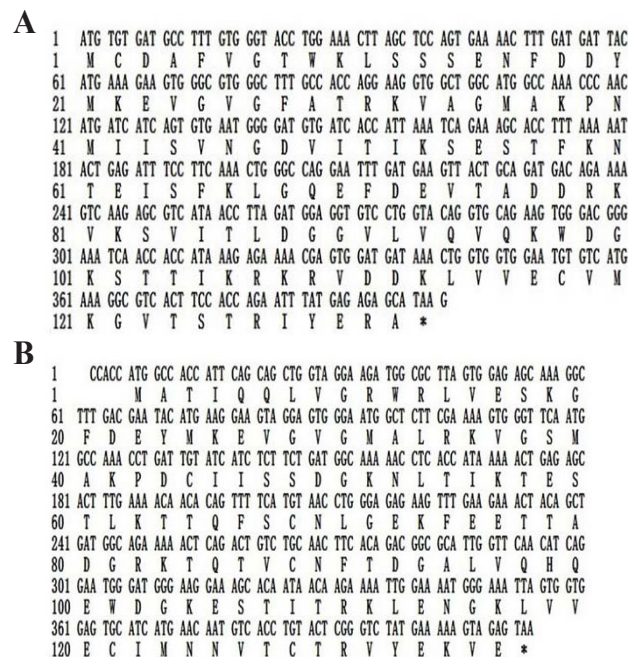


Figure 1. Nucleotide sequence of cDNA encoding the giant panda *FABP4* (A) and *FABP5* (B) and the amino acid sequence deduced from the open reading frame. Nucleotides are numbered in the 5'- to 3'-direction. The predicted amino acid sequence of the gene is shown under the nucleotide.

Base bias analysis showed that the content of individual bases differed in different codon positions; nucleotide G was the highest in the first position, T was the highest in the second position, while in the third position, the contents of T, G, and C were very similar and only A was the lowest. Furthermore, base substitution statistics suggested that the number of transitions was obviously higher than that of transversions, which is in accordance with the general rule that nucleotide substitution is the main mechanism of DNA evolution, and that nucleotide transition is the main mechanism of nucleotide substitution. In addition, variable sites and nucleotide substitutions in the third codon position were far higher than those in the first or second positions of codons, which suggested a high rate of nucleotide substitution in the third positions of codons during evolution.

Alignment analysis comparing cDNA sequences and deduced amino acid sequences of *FABP4* and *FABP5* from the giant panda and other mammals was performed using the DNASTar Lasergene software. This analysis indicated that both the nucleotide sequences and the deduced amino acid sequences are highly conserved (Tables 3 and 4). The giant panda shares the highest similarity in the nucleotide and amino acid sequences of *FABP4* with *H. sapiens* (93.5 and 95.5%, respectively), whereas it shares the highest similarity for nucleotide and amino acid sequences of *FABP5* with *B. taurus* (93.6 and 94.1%, respectively).

Table 3. Comparison of nucleotide and protein sequence of *FABP4* among 5 mammal species.

	<i>A. melanoleuca</i> (JN008911)	<i>H. sapiens</i> (BC003672)	<i>R. norvegicus</i> (BC084721)	<i>O. aries</i> (EU301804)	<i>B. taurus</i> (X89244)
<i>A. melanoleuca</i>	-	95.5%	87.9%	87.1%	84.1%
<i>H. sapiens</i>	93.5%	-	89.4%	86.4%	84.1%
<i>R. norvegicus</i>	83.0%	83.5%	-	84.1%	83.3%
<i>O. aries</i>	90.5%	89.5%	81.5%	-	93.2%
<i>B. taurus</i>	86.0%	89.5%	80.5%	96.0%	-

The homology matrix of *FABP4*-encoding sequence is below the diagonal, the homology matrix of protein sequence is above the diagonal.

Table 4. Comparison of nucleotide and protein sequence of *FABP5* among 5 mammal species.

	<i>A. melanoleuca</i> (JN008912)	<i>H. sapiens</i> (BC019385)	<i>R. norvegicus</i> (S83247)	<i>O. aries</i> (FJ546079)	<i>B. taurus</i> (BT02098)
<i>A. melanoleuca</i>	-	89.6%	76.3%	93.3%	94.1%
<i>H. sapiens</i>	91.7%	-	80.7%	89.6%	91.9%
<i>R. norvegicus</i>	78.9%	80.4%	-	76.3%	77.8%
<i>O. aries</i>	93.1%	90.0%	77.7%	-	97.8%
<i>B. taurus</i>	93.6%	90.4%	77.9%	97.5%	-

The homology matrix of *FABP5*-encoding sequence is below the diagonal, the homology matrix of protein sequence is above the diagonal.

Prediction and analysis of the FABP4 and FABP5 proteins

The molecular weight and theoretical isoelectric point (pI) of the putative FABP4 protein for the giant panda were determined to be 14.6949 kDa and 8.51, respectively, whereas those of the FABP5 protein were found to be 15.1333 kDa and 6.60, respectively. Physical and chemical analysis showed that the molecular weights of the two proteins were very similar, while some variance in the pI of the two proteins was evident among the mammals, which was due to differences in the degree of ionization of the amino group (-NH₂) and the carboxyl group (-COOH) (Tables 5 and 6).

Table 5. Molecular weight and isoelectric point of the FABP4 protein of giant panda and other mammal species.

	<i>A. melanoleuca</i>	<i>H. sapiens</i>	<i>M. musculus</i>	<i>B. taurus</i>	<i>O. aries</i>
Molecular weight (kDa)	14.6949	14.7188	14.6498	14.6778	14.6888
Isoelectric point	8.51	6.59	8.53	5.52	5.22

Table 6. Molecular weight and isoelectric point of the FABP5 protein of giant panda and other mammal species.

	<i>A. melanoleuca</i>	<i>H. sapiens</i>	<i>M. musculus</i>	<i>B. taurus</i>	<i>O. aries</i>
Molecular weight (kDa)	15.1333	15.1644	15.1374	15.0743	15.1033
Isoelectric point	6.60	6.60	6.14	7.58	7.58

Topology prediction revealed seven protein kinase C phosphorylation sites, two casein kinase II phosphorylation sites, two N-myristoylation sites, and one cytosolic fatty acid-binding protein signature in the FABP4 protein, and three N-glycosylation sites, three protein kinase C phosphorylation sites, one casein kinase II phosphorylation site, one N-myristoylation site, one amidation site, and one cytosolic fatty acid-binding protein signature in the FABP5 protein (Figure 2). Our analysis indicated minute differences in the functional sites among the reported mammalian proteins, but without exception. One cytosolic fatty acid-binding protein signature, which spans 19 amino acid residues, was found in the giant panda and other mammalian proteins, since the primary and most important role of this protein family is to bind long-chain fatty acids and other hydrophobic ligands. Although the 19 amino acid signature sequence is not exactly identical between the giant panda and other mammals, none of the changes result in alterations in the function or structure of the proteins.

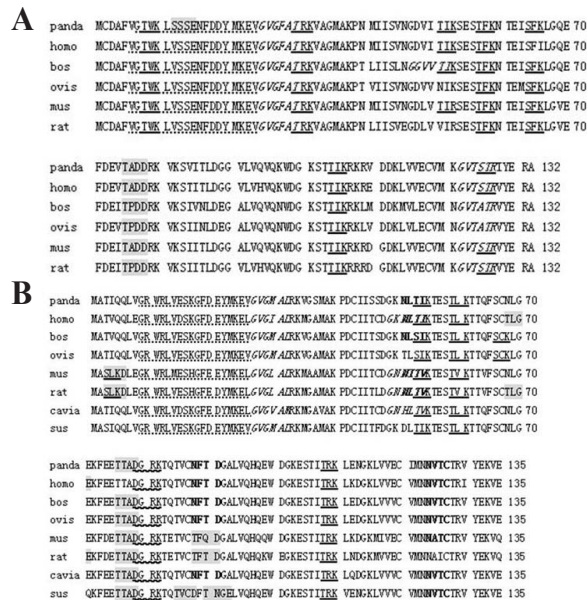


Figure 2. Comparison of the amino acid sequence of FABP4 (A) and FABP5 (B) among the giant panda and mammals reported. Panda = the giant panda; homo = *Homo sapiens*; bos = *Bos taurus*; ovis = *Ovis aries*; mus = *Mus musculus*; rat = *Rattus norvegicus*; cavia = *Cavia porcellus*; sus = *Sus scrofa*; bold = N-glycosylation site; italic = N-myristoylation site; solid underline = protein kinase C phosphorylation site; shaded = casein kinase II phosphorylation site; wave line = amidation site; dotted underline = cytosolic fatty acid-binding protein signature.

Overexpression of *FABP4* and *FABP5* in *E. coli*

FABP4 was overexpressed in *E. coli* using pET28a plasmids carrying strong promoter and terminator sequences derived from phage T7. For this purpose, the *FABP4* gene was amplified by PCR and cloned in a pET28a plasmid, resulting in a fusion gene coding for a protein bearing a His-tag extension at the N-terminus. Expression was tested by SDS-PAGE analysis of protein extracts from recombinant *E. coli* BL21 strains (Figure 3). Results indicated that the *FABP4* protein fused with the N-terminal His-tagged form gave rise to the accumulation of an expected 16.8-kDa polypeptide that formed inclusion bodies. Expression of the recombinant protein was evident after 30 min of induction and reached its highest level after 2 h. The *FABP5* gene was overexpressed by the same procedure, which produced a 17-kDa polypeptide (Figure 3).

These results suggested that the proteins were active and were the only proteins encoded by *FABP4* and *FABP5* from the giant panda. The expression products obtained could be used to purify these proteins in order to study their functions in more detail.

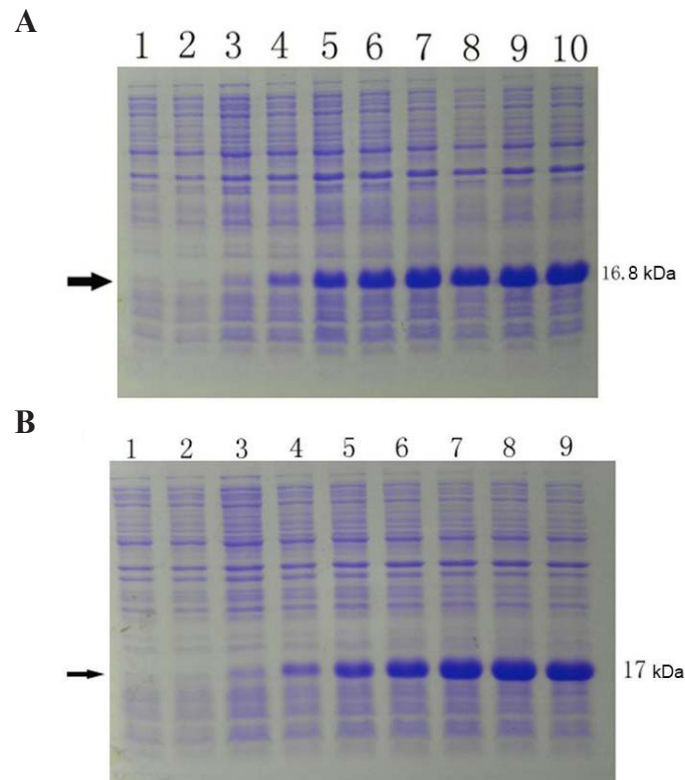


Figure 3. FABP4 (A) and FABP5 (B) proteins extracted from recombinant *Escherichia coli* strains were analyzed by SDS-PAGE stained with Coomassie blue R 250. Numbers on right show the molecular weight and arrows indicate the recombinant protein bands induced by IPTG with 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h (lanes 2-10) and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h (lanes 2-9), respectively; lane 1 = control.

DISCUSSION

FABPs belong to the conserved multigene family of intracellular lipid-binding proteins (Choromańska et al., 2011). These proteins are ubiquitously expressed in vertebrate tissues, with each FABP showing distinct expression patterns (Storch and McDermott, 2009). Various functions have been proposed for these proteins, including the promotion of cellular uptake and transport of fatty acids, the targeting of fatty acids to specific metabolic pathways, and participation in the regulation of gene expression and cell growth (Uysal et al., 2000).

FABP4 and *FABP5* are two closely related fatty acid-binding proteins that are expressed primarily in adipose tissue and/or macrophages. Alignment analysis of *FABP4* and *FABP5* of the giant panda and other mammalian species indicated that both the nucleotide and the deduced amino acid sequences are highly conserved. No deletions or insertions of nucleotides or amino acid residues were detected. Nonetheless, some polymorphic sites were identified in the deduced amino acid sequences of the *FABP4* and *FABP5* proteins. These polymorphic sites are located irregularly throughout the amino acid sequences, and have all resulted from the transversion or transition of the corresponding codons, without any deletion and insertion of bases. It is noteworthy that polymorphic sites located within the cytosolic fatty acid-binding protein signature sequences have not resulted in any changes in the functional role of binding long-chain fatty acids and other hydrophobic ligands.

Although some polymorphic sites are located within functional sites, indicating that these sites are not conserved, secondary and tertiary structure analysis showed that all amino acid sequences formed the same main structures (Figures 4, 5, and 6). For *FABP4*, residues spanning from 7 to 131 formed the same main structure: two helices (15-24, 26-36), and ten beta strands (6-14, 38-45, 54-47, 60-66, 70-74, 78-87, 90-97, 100-109, 112-119, 122-131). For *FABP5*, residues spanning from 2 to 133 formed the same main structure: three helices (3-8, 18-27, 29-39) and ten beta strands (9-17, 41-48, 50-58, 61-69, 71-77, 81-90, 92-100, 103-112, 114-122, 125-134). This fact shows that variation among sites has no effect on the structure and function of the *FABP4* and *FABP5* proteins. This may reflect an ancient evolutionary relationship between these proteins and the primary, ancient structural and functional feature of the FABP protein family.

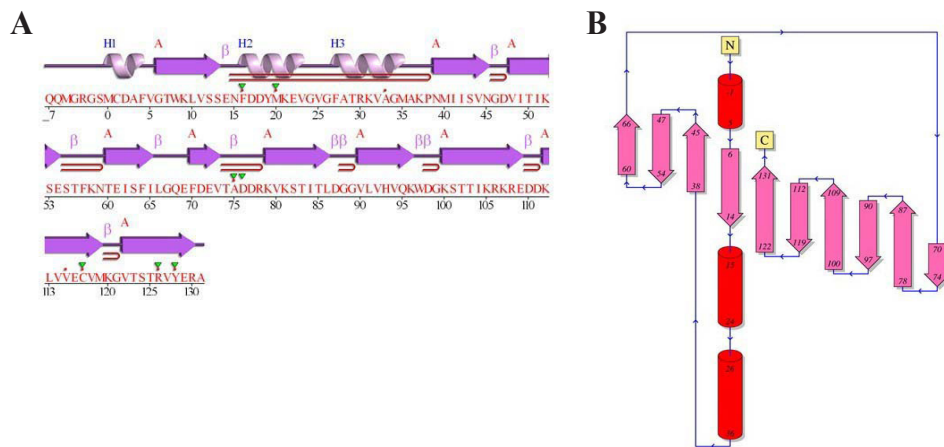


Figure 4. Secondary structure of *FABP4* of the giant panda. **A.** β = beta turn; H = helices; γ = gamma turn; hairpin loop = beta hairpin; A = beta strands. **B.** Pink thick arrow = beta strands; red cylinder = helices.

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