

A cDNA encoding acyl-CoA-binding protein in *Onychostoma macrolepis*: Molecular characterization and expression analysis

Xiaofei Yang¹, Shaogang Xu¹⁺, Yuezhi Wang¹ and Guiqiang Yang¹

¹ National Engineering Research Center for Freshwater Fisheries & Beijing Fisheries Research Institute

Abstract. To study the functions of ACBP in fish growth and metabolism, we isolated and characterized the cDNA from *Onychostoma macrolepis*. The full length *Oma-ACBP* cDNA(GB: JN634871) utilizing RACE method, which was 1582 bp in length, contains a 76bp 5'-UTR, an open reading frame of 1071 bp encoding a 357 amino acid and 432 bp 3'-UTR. The theoretical molecular weight was 39.2 kDa and isoelectric point was 6.78. The sequence analysis indicated that the *Oma-ACBP* had significant similarity across fishes, the sequence shared 93% identity with *Cyprinus carpio*. A phylogenetic tree was constructed showed that ACBP family protein sequences from fishes and vertebrates had high homology with *Oma-ACBP*, the sequence shared 93% identity with *Cyprinus carpio*. The qRT-PCR analysis demonstrated that the *Oma-ACBP* was widely detected in tissues and was mainly detected in ovary(p<0.05). In diet challenge, the *Oma-ACBP* transcripts showed a reduction from group 32% to 42% in spleen and intestine, a quickly growth from group 42% to 52% in spleen. The expression of *Oma-ACBP* was detected post starvation challenge, the expression was highly expressed at 2-day, 5-day and 9-day, the expression was gradually increased from 3-day to 5-day, and was decreased from 5-day to 8-day.

Keywords: ACBP, *Onychostoma macrolepis*, cloning, qRT-PCR, expression, diet challenge, starvation

1. Introduction

Acyl-CoA binding protein(ACBP) is highly conserved and considered to be the primary intracellular acyl-CoA binding protein, with molecular masses of 9-10 kDa cytosolic protein and consists of 86–103 amino acids[1, 2, 3]. ACBP was first isolated from rat brain and was named diazepam-binding inhibitor[4]. ACBP is protein able to bind long chain acyl-CoA esters(C14–C22) in a one-to-one binding mode with high specificity and affinity(Kd, 1–10 nM)[5, 6]. ACBP is involved in lipid metabolism, act as an acyl-CoA transporter and pool former[7]. ACBP plays a key role in binding steroidogenesis via to a specific outer mitochondrial membrane receptor and modulation of fatty acid biosynthesis[8]. ACBP was also apparently involved in regulation of the nature of innate immunity[9]. ACBPs possesses at least three major types: L-ACBP(first characterized in liver), T-ACBP(identified in testis) and B-ACBP(brain)[10, 11].

Now, the ACBP has been identified in many aquatic animals, such as *Strongylocentrotus intermedius* [12], *Perkinsus marinus*(GenBank number: XM002787996), *Xenopus laevis*[13], specifically, the ACBP has been identified in many fishes, such as *Ictalurus punctatus*[14], *Oncorhynchus nerka*[15], *Salmo salar*[16], *Danio rerio*[17], *Cyprinus carpio*[18], however, there is no report is available on the ACBP and its expression in the largescale shoveljaw fish, it is an omnivorous species belonging to the family *Cyprinidae* of the class *Varicorhinus*, and is a wild Chinese indigenous species[19]. The aim of the present study was to characterize the relationship between the expression of ACBP and the metabolism of lipid. We have isolated the *Oma-ACBP* cDNA, the analysis software was used to analyze the sequence of *Oma-ACBP*. The expression patterns following starvation and different dietary challenge were also analyzed in order to understand the functions in metabolism during the growth of largescale shoveljaw fish.

⁺ Corresponding author. Tel.: + 86-010-67565314.
E-mail address: xushaogang@bjfishery.com.

2. Materials and methods

2.1. Materials collection

Largescale shoveljaw fish (*Onychostoma macrolepis*) 17-25g in body weight, 12.3±3.2 cm in body length were obtained from National Engineering Research Center for Freshwater Fisheries in Fangshan District(Beijing, China), and were cultured in 0.5m³ tanks filled with air-pumped freshwater[19]. For the cloning and examination of *Oma-ACBP* in the organizations: the gonad, ovary, liver, heart, brain, cheek, intestine, muscle, eye, spleen were collected from the mature Largescale shoveljaw fish. In diet challenge experiments, the fishes were fed of diet at the protein content of 32%, 42% and 52%(20 fishes each group, totle of 60 fishes), following the 12-week feeding, all of the fishes were maintained in a concrete condition until 5-hour post feed, followed by samples collection from three random fish, the liver, muscle and intestine were collected and the expression of *Oma-ACBP* was investigated, the diet of Tubifex(*Limnodrilus hoffmeisteri*) as the control group. In the starvation challenge experiments, liver was collected at 0 to 14 day post-starvation[20]. All of samples were snap frozen in liquid nitrogen and stored at -80 °C until use.

2.2. RNA extraction

Total RNAs from a variety of tissues(gonad, ovary, liver, heart, brain, cheek, intestine, muscle, eye, spleen) were extracted and purified by the Trizol RNAPrep Kit(Tiagen, Beijing, China) following the manufacturer's instructions. The purity of RNAs were verified by measuring the absorbance at 230, 260, 280 and 320 nm by Smart specTMplus spectrophotometer(Bio-Rad, Hercules, US) and its quality and integrity was confirmed by 1% agarose electrophoresis. The RNA were reversed and transcribed to cDNAs using the Reverse Transcriptase kit(TaKaRa, Dalian, China).

2.3. The cDNA Cloning of *Oma-ACBP*

The sequences of primers used for cloning were listed in Table.1. By using primers PF1 and PR1 designed by the conserved regions of the ACBP genes from other fishes, a conservative fragment of 517 bp was amplified under the PCR condition: 94°C(30s), 55°C(30s), and 72°C(120s) for 35 cycles using a Eppendorf PCR cycler. By using primers: P3RACE-1, P3RACE-2, P5RACE-1, P5RACE-2, the 5' and 3' cDNA ends of *Oma-ACBP* was obtained by using the BD SMART RACE Kit(Clontech, Carlsbad, US) following the manufacturer's instructions. The amplified fragments were separated and purified with Axygen Gel Extraction Kit(Axygen, Hangzhou, China). The purified fragments were then cloned into pMD19-T vector and propagated in *E. coli* DH5 α (Takara, Dalian, China), and were sequenced by Tsingke BioTech company(BioTech company, Beijing, China).

Table.1 ACBP and β -actin gene of *Onychostoma macrolepis*, Corresponding PCR primers used for fragment cloning and quantitative real time PCR.

Primers	Sequence(5'→3')	Position	Product(bp)
For the cloning of conserved sequence			
PF1	gcttggcagttatattgtg	290-806	517
PR1	cttctggactctggccc		
For the cloning of 3' end RACE and 5' end RACE			
P3RACE-1	gtttcacacacccttcagtcag	766-1582	817
P3RACE-2	ggagtagagaagatggctaaagaat	599-1582	984
P5RACE-1	gggtctggaagccctttgtg	1-389	389
P5RACE-2	tgacagaatcgtctttgccagc	1-521	521
Primers used for quantitative real-time PCR			
P-QF	acatcaccaccatccgactc	411-700	290
P-QR	cggtccgtttatgactccaatc		
actinF	tcccctcaatcccaaagcc		121
actinR	ggagtccatcaccgataaccagt		

2.4. Sequence and Phylogenetic analysis of *Oma-ACBP*

Similarity analysis was performed by Primer Premier 5.0(Premier Biosoft International, Palo Alto, CA). The deduced amino acid sequence was obtained by using the tool of Open Reading Frame Finder(ORF

Finder, <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Sequence assembly was performed using a DNAMAN software (Lynnon Biosoft, US). The Molecular Evolution Genetics Analysis (MEGA) version 4.0 and ClustalW2 were used to construct a phylogenetic tree using the neighbor-joining method. The isoelectric point (pI) and molecular weight (MW) of protein were performed by ExPASy PeptideMass (<http://us.expasy.org/tools/peptide-mass.html>).

2.5. Quantitative real-time PCR (qRT-PCR) analysis of *Oma-ACBP*

First-strand cDNA was performed using PrimeScript[®]RT Enzyme (Takara, Dalian, China). Quantitative real-time PCR (qRT-PCR) was performed using *Oma-ACBP* realtime primers PQF and PQR and β -actin gene realtime primers actinF and actinR (Table.1) by Funglyn FTC-3000 cycler (Funglyn Biotech, Toronto, Canada). The total 20 μ l reaction system, mainly consisted of 10 μ l 2 \times SYBR Premix Ex Taq (Takara, Dalian, China), 2 μ l cDNA, 0.8 μ l each of 10 μ M forward and reverse primer, 0.4 μ l ROX and 6 μ l DNase-RNase free water. The real-time PCR program was 95 $^{\circ}$ C for 5s, followed by 45 cycles of 95 $^{\circ}$ C for 5s, 60 $^{\circ}$ C for 34s. Dissociation melting curves analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The relative quantification between ACBP and β -actin transcript were calculated using the comparative CT method.

3. Results

3.1. Cloning of the *Oma-ACBP*

Through RT-PCR and RACE methods, one ACBP was cloned from *Onychostoma macrolepis*. The full length *Oma-ACBP* cDNA (GenBank accession number: JN634871), which was 1582 bp in length, contained an open reading frame (ORF) of 1071 bp encoding a 357 amino acid polypeptide along with a 76bp 5'-UTR (5'-Untranslated Regions) and 432bp 3'-UTR with the polyA tail. Theoretical molecular weight of *Oma-ACBP* was 39.2 kDa and isoelectric point was 6.78. Multiple sequence alignment based on the deduced *Oma-ACBP* proteins of *Danio rerio*, *Cyprinus carpio*, *Salmo salar* and other five species showed that the *Oma-ACBP* had significant sequence similarity across fishes and had similarities with other species (Table 2, Fig.1).

Phylogenetic analysis of the identified ACBP family protein sequences from fishes and vertebrates indicated that *Oma-ACBP* had high homology with fishes, the sequence shared 93% identity with *Cyprinus carpio*, 90% sequenced identity with *Danio rerio*, and 80% with *Salmo salar*. One group consisted of Fish ACBP (*Danio rerio*, *Cyprinus carpio* and *Salmo salar*), mammal ACBP (*Mus musculus*, *Bos Taurus*, *Homo sapiens*, *Ornithorhynchus anatinus*), which was consistent with the result of sequence alignment analysis, however, the relationship between fishes and vertebrates was more distant (Fig.2).

Table.2 Protein similarities of *Oma-ACBP* with other species (BLASTX).

Species	Score	Expected value	Identities (%)	Positives (%)	GenBank No.
<i>Danio rerio</i>	544	0	90%	96%	AAH76531
<i>Cyprinus carpio</i>	538	0	93%	94%	AAC19408
<i>Salmo salar</i>	520	0	80%	90%	ACN11335
<i>Gallus gallus</i>	415	2e-139	65%	80%	XP418965
<i>Xenopus laevis</i>	412	7e-139	68%	82%	AAI06605
<i>Meleagris gallopavo</i>	411	1e-138	66%	81%	XP003204816
<i>Anolis carolinensis</i>	409	5e-138	66%	81%	XP003223610
<i>Bos taurus</i>	405	9e-136	62%	76%	NP001029414

3.2. qRT-PCR analysis of *Oma-ACBP* expression

The expression in tissues was demonstrated that *Oma-ACBP* was detected in gonad, ovary, liver, heart, brain, cheek, intestine, muscle, eye, spleen, *Oma-ACBP* was mainly detected in ovary ($p < 0.05$, Fig.3). To detect the expression levels of *Oma-ACBP* during diet and starvation challenge in tissues, we performed a general overview on the expression pattern of *Oma-ACBP* in the whole challenge stages. In diet challenge (dietary protein content of 32%, 42% and 52%), the *Oma-ACBP* transcripts showed a reduction

Fig. 3: Quantitative real-time PCR analysis of *Oma-ACBP* expression in Tissues. The β -actin gene is applied as internal standard control. Data represent the means \pm SEM(n=3).

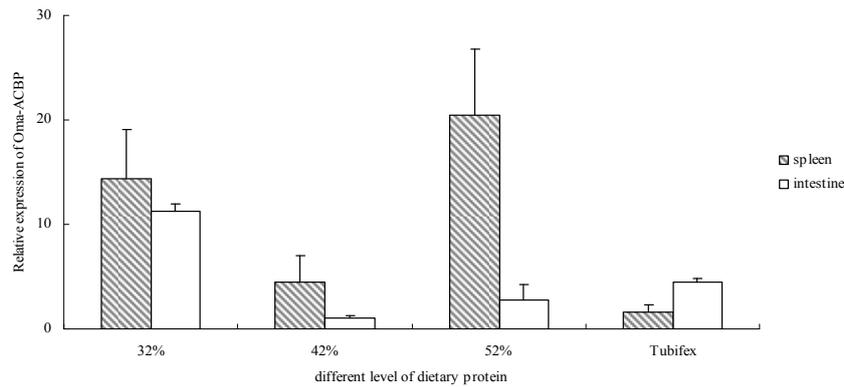


Fig. 4: Quantitative real-time PCR analysis of *Oma-ACBP* expression after diet challenge which different level of dietary protein(32%, 42% and 52%, Tubifex as the control group)

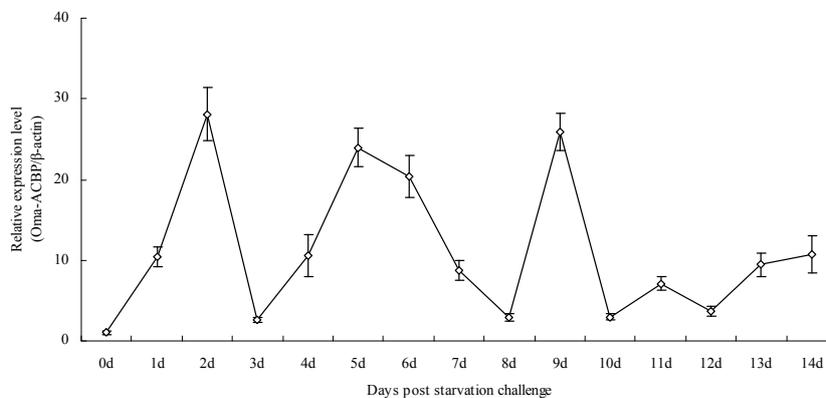


Fig. 5: Quantitative real-time PCR analysis of *Oma-ACBP* expression post starvation challenge from 0-day to 14-day.

4. Results Discussion

ACBP family proteins are evolutionary highly conserved acyl-CoA esters-bound proteins that are involved in lipid metabolism, transport acyl-CoA, fatty acid biosynthesis and showed antibacterial activity[6, 7, 21]. In order to clarify the mechanism of ACBP in *Onychostoma macrolepis*, we cloned *Oma-ACBP* full length cDNA, and analyzed its expression patterns in diet and starvation challenge. In the present study, we isolated and characterized member of the Acyl-CoA binding protein(ACBP), from *Onychostoma macrolepis* for the first time, the *Oma-ACBP* cDNA encodes a protein of 357 amino acids that show 80–90% identities with known ACBPs reported previously in fishes, the theoretical molecular weight was 39.2 kDa and isoelectric point was 6.78. Phylogenetic inference indicates that *Oma-ACBP* is phylogenetically closer to those of *Cyprinus carpio*, *Danio rerio* and *Salmo salar*, suggesting that ACBP is conserved in fish evolution and may have common functions in different species.

Quantitative real-time PCR analysis showed that *Oma-ACBP* was expressed in all tissues we tested and predominantly expressed in ovary. In *Helicoverpa armigera*, Northern and Western blot analyses of various tissues demonstrated that ACBP was highly expressed in the midgut and not detected in the epidermis, head-thorax, and hemocytes[22]. In *Strongylocentrotus internedius*, ACBP was highly expressed in gonads, but weakly expressed in muscles, coelomic fluid and tube feet[20]. Given these results, we presumed that *Oma-ACBP* might be ubiquitously expressed in all tissues, the tissue specific regulation of *Oma-ACBP* expression leads to the hypothesis that *Oma-ACBP* may have different functions in tissues.

In rats, feeding of a high-fat diet for 48 hours caused ACBP levels to increase[23]. In Atlantic cod, the effect of two different diets(fish meal, FM; dehulled and extracted soybean meal, SBM) on gene expression

in the intestine was studied, expression of four genes were significantly up regulated in intestine of cod fed SBM compared to cod fed FM[24]. In regulation of lipid metabolism, ACBP can act as transport acyl-CoA of long chain esters[25]. In *Strongylocentrotus internedius*, ACBP was highly expressed in gonads of female sea urchins but weakly expressed in gonads of male urchins feeding *Ulva pertusavar*[20]. In rainbow trout, the ACBP expression changes in muscle and liver between domesticated species and wild species, the ACBP of domesticated fish predominantly for the regulation and control[26]. In this study, the diet challenge(die at the protein content of 32%, 42% and 52%) was set up, we chose the Tubifex as the diet control group for determination of the gene expression, the *Oma-ACBP* transcripts showed a reduction from group 32% to 42% in spleen and intestine, a steep growth from group 42% to 52% in spleen. The observation suggested that the expression of *Oma-ACBP* exhibited much stronger in spleen than intestine.

In *Strongylocentrotus internedius*, the gene expression in gonads female urchins was weak after 2-day starvation, turned to high after 4-day starvation, and kept high from 13-day to 30-day starvation[20]. Another study led to the identification of novel genes and proteins that responded to long term feeding with a high energy/high fat diet[27]. In the present study, the relationship of *Oma-ACBP* expression with different conditions in metabolism implies its importance for cellular function, *Oma-ACBP* might be expected to play a irreplaceable role in lipid and energy metabolism.

In conclusion, this study characterized the ACBP(GenBank accession number: JN634871) gene from *Onychostoma macrolepis*, the *Oma-ACBP* expressed in various tissues, and distinct expression pattern appeared in diet and starvation challenge. Thus, the *Oma-ACBP* should have a physiological and biochemical functions in energy metabolism, and might be expected play roles in activating the transcription of genes of the lipid and glucose metabolism.

5. Acknowledgements

This work was financially supported by the Beijing Municipal Science & Technology Commission (Z090605006009015), China. We gratefully thank Dr. Hongxia Hu and Dr. Zhaohui Tian for supplying their technical assistance.

6. References

- [1] M.-L. Chye, B.-Q. Huang and S. Y. Zee. Isolation of a gene encoding Arabidopsis membrane associated acyl-CoA binding protein and immunolocalization of its gene product. *The Plant Journal*. 1999, 18(2): 205-214.
- [2] N. J. Faergeman and J. Knudsen. Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. *Biochem. J*. 1997, 323(1): 1-12.
- [3] M. Liu, T.-Y. Zhang and W.-H. Xu. A cDNA encoding diazepam-binding inhibitor/acyl-CoA-binding protein in *Helicoverpa armigera*: Molecular characterization and expression analysis associated with pupal diapause. *Comp. Biochem. Phys. C*. 2005, 141(2): 168-176.
- [4] A. Guidotti, C. M. Forchetti, M. G. Corda, D. Konkel, C. D. Bennett, and E. Costa. Isolation, characterization and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. *Proc. Natl. Acad. Sci*. 1983, 80(11): 3531-3535.
- [5] M. Burton, T. M. Rose, N. J. Faergeman, and J. Knudsen. Evolution of the acyl-CoA binding protein(ACBP). *Biochem. J*. 2005, 392(2): 299-307.
- [6] B. B. Kragelund, J. Knudsen and F. M. Poulsen. Acyl-coenzyme A binding protein(ACBP), *BBA-Mol. Cell. Biol. L*. 1999, 1441(2-3): 150-161.
- [7] S. Mandrup, N. J. Faergeman and J. Knudsen. Structure, function and phylogeny of acyl-CoA binding protein. In: A. K. Duttaroy, F. Spener, editors. *Cellular Proteins and Their Fatty Acids in Health and Disease*. Weinheim: Wiley-VCH gmbH & Co. Kaga; 2004, 151-172.
- [8] V. Papadopoulos, A. Berkovich, K. E. Krueger, E. Costa and A. Guidotti. Diazepam binding inhibitor and its processing products stimulate mitochondrial steroid biosynthesis via an interaction with mitochondrial benzodiazepine receptors. *Endocrinology*. 1991, 129(3): 1481-1488.
- [9] Q. Ren, Z.-Q. Du, X.-F. Zhao and J.-X. Wang. An acyl-CoA-binding protein(FcACBP) and a fatty acid binding

protein(FcFABP) respond to microbial infection in Chinese white shrimp, *Fenneropenaeus chinensis*. Fish Shellfish Immun. 2009, 27(6): 739-747.

- [10] G. P. Owens, A. K. Sinha, J. M. Sikela, W. E. Hahn. Sequence and expression of the murine diazepam binding inhibitor. Mol. Brain. Res. 1989, 6(2-3): 101-108.
- [11] M. Metzner, K. P. Ruecknagel, J. Knudsen, G. Kuellertz, F. Mueller-Uri and B. Dietrich. Isolation and characterization of two acyl-CoA-binding proteins from proembryogenic masses of *Digitalis lanata* Ehrh. Planta. 2000, 210(4): 683–685.
- [12] Y. Q. Chang, J. Ding and X. F. Yang. The cDNA cloning and mRNA expression of ACBP gene in *Strongylocentrotus intermedius*. Unpublished.
- [13] S. L. Klein, R. L. Strausberg, L. Wagner, J. Pontius, S. W. Clifton and P. Richardson. Genetic and genomic tools for *Xenopus* research: The NIH *Xenopus* initiative. Dev. Dynam. 2002, 225(4): 683-685.
- [14] F. Chen, Y. Lee, Y. L. Jiang, S. L. Wang, E. Peatman and J. Abernathy, et al. Identification and characterization of full-length cDNAs in channel catfish(*Ictalurus punctatus*) and blue catfish(*Ictalurus furcatus*). PLoS. ONE. 2010, 5(7): E11546.
- [15] C. M. Elfstrom, C. T. Smith and J. E. Seeb. Thirty-two single nucleotide polymorphism markers for high-throughput genotyping of sockeye salmon. Mol. Ecol. Notes. 2006, 6(4): 1255-1259.
- [16] H. Takle, G. Baeverfjord and O. Andersen. Identification of temperature sensitive genes in Atlantic salmon(*Salmo salar L*) embryos by RNA Arbitrarily Primed(RAP)-PCR. Unpublished.
- [17] R. L. Strausberg, E. A. Feingold, L. H. Grouse, J. G. Derge, R. D. Klausner and F. S. Collins, et al. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. P. Natl. Acad. SCI. USA. 2002, 99(26): 16899-16903.
- [18] J. L. Chang and H. J. Tsai. Carp cDNA sequence encoding a putative diazepam-binding inhibitor/endozepine/acyl-CoA-binding protein. BBA-Protein Struct. M. 1996, 1298(1): 9-11.
- [19] X. F. Yang, S. G. Xu, Y. Z. Wang, G. Q. Yang. Cloning, characterization and expression analysis of DEAD-box family vasa gene, in Largescale shoveljaw fish(*Onychostoma macrolepis*), IEEE US, vol.1, 237-241, May 2011[The 5th International Conference on Bioinformatics and Biomedical Engineering].
- [20] X. F. Yang, Y. Q. Chang, H. Han. Acyl-CoA binding protein(ACBP) encoding genes in the sea urchin *Strongylocentrotus internedius*, International Marine Biotechnology Association(IMBA). Qingdao, China, vol. abstracts, pp. 67, October 2010[The 9th International Marine Biotechnology Conference].
- [21] B. Agerberth, A. Boman, M. Andersson, H. Jörnvall, V. Mutt and H. G. Boman. Isolation of three antibacterial peptides from pig intestine: gastric inhibitory polypeptide(7-42), diazepam binding inhibitor(32-86) and a novel factor, peptide 3910. Eur. J. Biochem. 1993, 216(2): 623-629.
- [22] J. L. Wang, J.-X. Wang and X.-F. Zhao. Molecular Cloning and Expression Profiles of the Acyl-CoA-Binding Protein Gene From the Cotton Bollworm *Helicoverpa armigera*. Arch. Insect. Biochem. 2008, 68(2): 79-88.
- [23] N. J. Færgeman, M. Wadum, S. Feddersen, M. Burton, B. B. Kragelund and Jens Knudsen. Acyl-CoA binding proteins, tructural and functional conservation over2000 MYA. Mol. Cell. Biochem. 2007, 299(1-2): 55-65.
- [24] E. Lilleeng M. K. Frøystad, K. Vekterud, E. C. Valen and Å. Krogdahl. Comparison of intestinal gene expression in Atlantic cod(*Gadus morhua*) fed standard fish meal or soybean meal by means of suppression subtractive hybridization and real-time PCR. Aquaculture. 2007, 267(1-4): 269-283.
- [25] T. Helledie, L. Grøntved, S. S. Jensen, P. Kiilerich, L. Rietveld and T. Albrektsen, et al. The gene encoding the Acyl-CoA-binding protein is activated by peroxisome proliferator-activated receptor c through an intronic response element functionally conserved between humans and rodents. J. Biol. Chem. 2002, 277(30): 26821-26830.
- [26] W. Tymchuk, D. Sakhrani and R. Devlin. Domestication causes large-scale effects on gene expression in rainbow trout: Analysis of muscle, liver and brain transcriptomes. Gen. Comp. Endocr. 2009, 164(2-3): 175-183.
- [27] C.-I. Kolditz, G. Paboef, M. Borthaire, D. Esquerré, M. SanCristobal and F. Lefèvre, et al. Changes induced by dietary energy intake and divergent selection for muscle fat content in rainbow trout(*Oncorhynchus mykiss*), assessed by transcriptome and proteome analysis of the liver. BMC. Genomics. 2008, 9(506): 47-64.