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Molecular cloning and expression analysis of the full-length cDNA of grass carp acetyl-CoA carboxylase gene (Postprint)

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Abstract

This study employed reverse transcription PCR (RT-PCR) and rapid amplification of cDNA ends (RACE) techniques to clone the full-length cDNA of acetyl-CoA carboxylase (ACC2) gene from grass carp, and used real-time quantitative PCR to investigate the expression of ACC2 gene in various tissues including hepatopancreas, spleen, brain, foregut, midgut, hindgut, kidney, muscle, heart, and mesenteric adipose tissue. Additionally, the expression changes of ACC2 mRNA in hepatopancreas and muscle of grass carp fed different dietary lipid sources for 12 weeks, as well as in hepatopancreas at 3, 6, 12, and 24 h after refeeding following starvation, were examined. The results showed that the full-length cDNA of grass carp ACC2 gene was 7,533 bp, containing a 7,149 bp open reading frame encoding 2,382 amino acids. The calculated molecular mass of ACC2 protein was 268.34 ku with an isoelectric point of 6.13. Alternative splicing was observed in grass carp ACC2 gene, generating another isoform lacking 8 amino acids compared with the 268.34 ku ACC2 protein. ACC2 gene was expressed in all examined tissues, with the highest relative expression level of ACC2 mRNA in muscle (29.13) and the lowest in hepatopancreas (1.90). The relative expression level of ACC2 mRNA in muscle showed no significant difference from that in brain, foregut, and heart ($P>0.05$), but was significantly higher than in other tissues ($P<0.05$). Different dietary lipid sources had no significant effect on the relative expression level of ACC2 mRNA in hepatopancreas and muscle of grass carp ($P>0.05$). After refeeding following starvation, the relative expression level of ACC2 mRNA in hepatopancreas peaked at 12 h (6.17) and then decreased markedly to only 2.84 at 24 h. This study successfully cloned the full-length cDNA of grass carp ACC2 gene, whose major functional sites, including the ATP-binding site and biotin-binding site, were basically conserved compared with other vertebrates. The grass carp ACC2 gene was mainly expressed in tissues active in lipid catabolism such as muscle; different dietary lipid sources

had no significant effect on the relative expression level of ACC2 mRNA in hepatopancreas of grass carp; and after refeeding following starvation, the relative expression level of ACC2 mRNA in hepatopancreas was highest at 12 h post-feeding.

Full Text

Preamble

Molecular Cloning and Expression Analysis of Full-Length Acetyl-CoA Carboxylase cDNA from Grass Carp (Ctenopharyngodon idella)

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Abstract: The full-length cDNA of acetyl-CoA carboxylase (ACC2) was cloned from grass carp using reverse transcription PCR (RT-PCR) and rapid amplification of cDNA ends (RACE) techniques. Real-time quantitative PCR was employed to investigate ACC2 gene expression across various tissues including hepatopancreas, spleen, brain, anterior intestine, mid-intestine, posterior intestine, kidney, muscle, heart, and mesenteric adipose tissue. Additionally, we examined ACC2 mRNA expression changes in hepatopancreas and muscle of grass carp fed different lipid source diets for 12 weeks, as well as in hepatopancreas at 3, 6, 12, and 24 hours after starvation and refeeding. The results revealed that the full-length ACC2 cDNA spans 7,533 bp, containing a 7,149 bp open reading frame that encodes 2,382 amino acids. The calculated molecular mass of ACC2 protein is 268.34 kDa with an isoelectric point of 6.13. Alternative splicing was identified in the grass carp ACC2 gene, generating another isoform lacking eight amino acids compared to the 268.34 kDa ACC2 protein. ACC2 was expressed in all examined tissues, with the highest mRNA level detected in muscle (29.13) and the lowest in hepatopancreas (1.90). Muscle ACC2 mRNA expression showed no significant difference from brain, anterior intestine, and heart ($P>0.05$) but was significantly higher than other tissues ($P<0.05$). Different dietary lipid sources had no significant effect on ACC2 mRNA expression in hepatopancreas or muscle ($P>0.05$). Following starvation and refeeding, hepatopancreas ACC2 mRNA expression peaked at 12 hours (6.17) then declined markedly to 2.84 at 24 hours. This study successfully cloned the full-length ACC2 cDNA from grass carp, revealing that key functional sites including ATP-binding and biotin-binding sites are largely conserved compared to other vertebrates. The ACC2 gene is predominantly expressed in tissues with active lipolysis such as muscle, and its hepatopancreas expression is not significantly affected by dietary lipid sources, reaching maximum expression 12 hours after refeeding.

Keywords: grass carp; acetyl-CoA carboxylase ; molecular cloning; gene expression

Acetyl-CoA carboxylase (ACC) is the rate-limiting enzyme in fatty acid synthesis, using biotin as a cofactor to catalyze the conversion of acetyl-CoA to malonyl-CoA (MA), thereby providing substrates for fatty acid synthesis. In prokaryotes, ACC comprises three subunits encoded by separate genes: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT). In eukaryotes, a single gene encodes all three functional domains. Two ACC isoforms exist: ACC1 (or ACC) and ACC2 (or ACC), encoded by ACACA and ACACB genes respectively. These isoforms exhibit distinct tissue distributions—ACC1 is predominantly expressed in lipogenic tissues such as liver, adipose tissue, and mammary gland to supply fatty acid synthesis, whereas ACC2 is mainly expressed in tissues with active fatty acid oxidation like muscle and heart, where it regulates carnitine palmitoyltransferase-I (CPT-I) activity. In homeothermic animals, full-length cDNAs of ACC1 have been cloned from rat and cattle, while human ACC2 cDNA contains a 7,449 bp open reading frame encoding 2,483 amino acids and maps to chromosome 12q23. ACC2 shares 75% amino acid sequence similarity with ACC1, with the primary difference residing in the N-terminus where mammalian ACC2 contains approximately 140 additional amino acids that target it to the mitochondrial outer membrane. Research on fish ACC genes remains limited, though the full-length ACC1 cDNA has been cloned from grass carp.

Grass carp (Ctenopharyngodon idella), belonging to Cypriniformes and Cyprinidae, represents an important aquaculture species in China due to its rapid growth, palatable flesh, diverse feed sources, and economic value. However, intensive aquaculture expansion has led to excessive hepatic lipid accumulation and associated metabolic disorders in farmed fish. With rapid advances in molecular biology, investigating nutritional regulation mechanisms at the molecular level represents a new frontier in nutrition research. This study aimed to clone the full-length ACC2 cDNA from grass carp, analyze its tissue expression patterns, and examine ACC2 mRNA expression changes in muscle and hepatopancreas following different dietary lipid sources and in hepatopancreas during starvation-refeeding cycles. These investigations will elucidate the role of ACC2 in lipid metabolism regulation, provide references for optimizing grass carp feed formulations, and offer theoretical support for addressing nutritional fatty liver disease in aquaculture.

Materials and Methods

Experimental Fish

Experimental fish were purchased from Huandun Fishery in Ganyu District, Lianyungang, with an average weight of (56.4±1.8) g per fish.

Experimental Primers

All primers used in this study are listed in . Three pairs of ACC2 real-time quantitative PCR primers were designed, and two pairs of primers were designed for each candidate reference gene including -actin (ACTB), eEF1A, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)1, GAPDH2, RPL13A, and TUBB2. Primers with amplification efficiencies between 0.90 and 1.05 were selected for final quantification.

Total RNA Extraction and First-Strand cDNA Synthesis

Six grass carp were sacrificed 6 hours post-feeding. Tissues including hepatopancreas, spleen, kidney, anterior intestine, mid-intestine, posterior intestine, mesenteric adipose tissue, brain, white muscle, and heart were rapidly dissected and snap-frozen in liquid nitrogen. Total RNA was extracted from each tissue using the QIAGEN RNeasy Lipid Tissue Mini Kit according to the manufacturer' s protocol. RNA integrity was assessed by 1% agarose gel electrophoresis, and concentration was determined using a nucleic acid-protein quantifier. First-strand cDNA was synthesized using the QIAGEN reverse transcription kit with Oligo(dT)16AP primers, following the recommended procedures for DNA contamination removal.

ACC2 Gene cDNA Cloning

Based on zebrafish (Danio rerio) (XM_009301377) and previously amplified grass carp sequences, four pairs of degenerate primers and four pairs of specific primers were designed to amplify eight core fragments of the ACC2 gene. PCR was performed in a 25 L reaction system containing 12.5 L Sapphire Amp Fast PCR Master Mix (TaKaRa), 1 L mixed first-strand cDNA from hepatopancreas and heart, 0.5 L each of forward and reverse primers, and 10.5 L double-distilled water (ddH2O). Amplification conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were examined by 1% agarose gel electrophoresis, gel-purified, cloned, and sequenced.

3' and 5' RACE of Grass Carp ACC2 Gene

Based on the core sequences of ACC2 gene, specific primers for 3' and 5' RACE were designed (). The 3' and 5' ends were cloned following the method described by Cheng et al.

Sequence Analysis

The full-length ACC2 gene was assembled from core sequences and 3' and 5' end sequences using SeqMan software from the DNAstar 7.1 package. Signal

peptides were predicted using http://www.cbs.dtu.dk/services/SignalP/, and protein domains were analyzed via http://smart.embl-heidelberg.de/.

Tissue Expression of Grass Carp ACC2 Gene

Experimental fish were purchased from Huandun Fishery in Ganyu District, Lianyungang, with an average weight of (56.4±1.8) g. Six grass carp were sacrificed, and tissues including hepatopancreas, spleen, kidney, anterior intestine, mid-intestine, posterior intestine, mesenteric adipose tissue, brain, white muscle, and heart were rapidly dissected and snap-frozen in liquid nitrogen. Total RNA was extracted from each tissue and reverse-transcribed into first-strand cDNA using random primers after DNA contamination removal. SYBR Green RT-qPCR was performed on a Step One Plus PCR system (ABI) using the QuantiNova SYBR Green PCR Kit (QIAGEN). Each sample was run in triplicate. Candidate reference genes included ACTB, eEF1A, GAPDH1, GAPDH2, RPL13A, and TUBB2. Reference genes were selected using the geNorm algorithm in GenEx 6.0.1 software for quantitative analysis of ACC2 tissue expression.

Experimental Diets

Three experimental groups were established with dietary lipid sources of 5% fish oil (fish oil group), 5% lard oil (lard oil group), and 5% soybean oil (soybean oil group). All other dietary components were identical (). Major ingredients including fish meal, fish oil, and premix were provided by Yuetai Group. Ingredients were weighed, mixed thoroughly for 10 minutes, and 500 mL water was added per kilogram of mixture. After kneading for 15 minutes, pellets were produced using a portable meat grinder, dried at 55°C for 3 hours, cooled at room temperature for 1 hour, sealed in bags, labeled, and stored for later use.

Feeding Management

The feeding trial was conducted in a single-cycle controlled experimental aquaculture system consisting of nine 240 L tanks. A total of 180 grass carp with average weight of (56.4±1.8) g were purchased from Huandun Fishery and randomly divided into three groups with three replicates each (20 fish per tank). The 12-week trial was maintained at 25–28°C. Fish were acclimated with commercial feed for one week to train them to surface feed before gradual transition to experimental diets. During the trial, fish were fed four times daily at 08:00, 11:00, 14:00, and 17:00. Initial daily feeding rate was set at 2% of body weight (25 g/tank) and adjusted based on consumption. Daily feed intake and feeding behavior were recorded, with feeding amount gradually increased to 35 g/tank.

Effects of Different Dietary Lipid Sources on ACC2 Gene Expression

After the feeding trial, remaining fish were counted and weighed individually. Three fish per tank (nine per group) were sacrificed for visceral somatic index

calculation and total RNA extraction from hepatopancreas and muscle for ACC2 expression analysis.

Effects of Starvation and Refeeding on ACC2 Gene Expression

Following the feeding trial, fish were fasted for 24 hours then refed. Three fish per tank (nine per group) were sacrificed at 3, 6, 12, and 24 hours post-refeeding for hepatopancreas collection, total RNA extraction, and ACC2 expression quantification.

Calculation Formulas for Related Indices

Weight gain rate (%) = 100 × (final mean body weight - initial mean body weight) / initial mean body weight
Feed coefficient = total feed intake per tank / total weight gain per tank
Visceral somatic index (%) = 100 × visceral weight / final body weight

Data Analysis

Reference genes were selected using the geNorm algorithm in GenEx 6.0.1 software. ACC2 relative expression levels were calculated using the 2-ΔΔCt method and expressed as mean ± standard deviation. Statistical analysis was performed using SPSS 17.0 software. All data passed normality and homogeneity of variance tests, allowing inter-group comparisons using one-way ANOVA with LSD post-hoc test. $P<0.05$ was considered statistically significant.

Results

Molecular Characteristics of Grass Carp ACC2 Full-Length cDNA

The full-length ACC2 cDNA from grass carp spans 7,533 bp, containing a 7,149 bp open reading frame encoding 2,382 amino acids. The calculated molecular mass is 268.34 kDa with an isoelectric point of 6.13, designated as ACC -1 (GenBank accession: MF611923). Alternative splicing generated another isoform, ACC -2 (GenBank accession: MF611924), with a molecular mass of 267.53 kDa, lacking eight amino acids compared to ACC -1.

Tissue Expression of Grass Carp ACC2 Gene

Using RPL13A and eEF1A as reference genes, RT-qPCR analysis revealed ACC2 expression in all ten examined tissues. The highest ACC2 mRNA expression was detected in muscle (29.13), followed by brain (19.45), anterior intestine (15.33), and heart (14.40), while hepatopancreas showed the lowest expression (1.90). Muscle ACC2 mRNA levels were not significantly different from brain, anterior intestine, and heart ($P>0.05$) but were significantly higher than all other tissues ($P<0.05$, [Figure 1: see original paper]).

Effects of Different Dietary Lipid Sources on Growth Indices

Growth performance was assessed at the end of the feeding trial. As shown in , weight gain rates in fish oil (139.6%) and soybean oil (138.8%) groups were significantly higher than in the lard oil group (128.8%), indicating fish oil and soybean oil are superior lipid sources for grass carp. Feed coefficients in fish oil (1.14) and soybean oil (1.17) groups were significantly lower than in the lard oil group (1.24), confirming lard oil is unsuitable as the sole lipid source.

Effects of Different Dietary Lipid Sources and Starvation/Refeeding on ACC2 Gene Expression

Analysis using six reference genes identified RPL13A and GAPDH2 as optimal references for different lipid source groups, while ACTB and GAPDH1 were selected for starvation/refeeding groups. Different dietary lipid sources did not significantly affect ACC2 mRNA expression in hepatopancreas or muscle ($P>0.05$, [Figure 2: see original paper]). However, starvation and refeeding significantly increased hepatopancreas ACC2 mRNA expression at 12 hours post-feeding ($P<0.05$), peaking at 6.17 before declining significantly to 2.84 at 24 hours ([Figure 3: see original paper]).

Discussion

Structure of Grass Carp ACC2 Protein

Functional domain analysis of grass carp ACC2 amino acids is presented in [Figure 4: see original paper]. Grass carp ACC2 is encoded by a single gene containing three domains: BC, BCCP, and CT, which constitute the three subunits. The glycine-rich region (GGGGKG) is considered the ATP-binding domain. The BC domain of grass carp ACC2 contains an ATP-binding site at Gly371-376, identical to those in grass carp ACC1, half-smooth tongue sole, and humans, indicating high conservation. The conserved Met-Lys-Met sequence is recognized as the biotin-binding site. The BCCP domain of grass carp ACC2 contains two tetrapeptide sequences, Val-Met-Lys-Met and Arg-Met-Lys-Met, located at amino acid residues 840-843 and 1698-1701, respectively. Studies have shown that biotin upregulates ACC2 expression in the hypothalamus, and excess biotin suppresses food intake in mice.

Tissue Expression of Grass Carp ACC2 Gene

Grass carp ACC2 was expressed in all ten examined tissues, with highest expression in muscle and lowest in hepatopancreas—15.3-fold higher in white muscle than hepatopancreas ([Figure 1: see original paper]). Similar to reports in humans showing high ACC2 expression in heart and skeletal muscle, and studies demonstrating predominant ACC2 expression in oxidative tissues like skeletal muscle and heart in rats, our results confirm that grass carp ACC2 is primarily expressed in muscle, consistent with other mammals. Since ACC2 product

MA is a potent CPT-I inhibitor, ACC2-deficient mammals show significantly reduced MA levels in heart and skeletal muscle. Other studies confirm that ACC2 activity determines MA levels in the heart, further demonstrating that ACC2 regulates fatty acid oxidation in skeletal muscle and heart.

Effects of Different Dietary Lipid Sources on Growth Indices

No significant differences in visceral somatic index were observed among groups, likely due to low dietary lipid levels. Fish oil and soybean oil groups showed significantly higher weight gain rates and lower feed coefficients compared to the lard oil group, indicating both are excellent lipid sources for grass carp with no significant difference between them, while lard oil is unsuitable as the sole lipid source. Zhang et al. reported that bullfrogs fed diets containing fish oil, soybean oil, or palm oil showed higher weight gain rates with no significant differences among these groups, all significantly outperforming those fed poultry fat or lard oil. Chen et al. found that juvenile red tilapia fed diets with 4% soybean oil or soybean oil-fish oil mixture showed significantly higher final body weight and weight gain rate compared to the 4% lard oil group, which had significantly higher feed coefficient. Cheng et al. demonstrated that grass carp fed lard oil accumulated more fat in hepatopancreas, muscle, and mesenteric adipose tissue with potential fatty liver lesions, while soybean oil-fed fish maintained normal hepatopancreas morphology, confirming soybean oil as superior. Liu et al. reported that mixed oil and fish liver oil produced the best growth performance in grass carp, with soybean oil and lard oil showing intermediate effects, consistent with our findings.

Effects of Different Dietary Lipid Sources on ACC2 Expression in Hepatopancreas and Muscle and Effects of Starvation/Refeeding

Feeding grass carp with fish oil, soybean oil, or lard oil for 12 weeks did not significantly affect ACC2 mRNA expression in hepatopancreas or muscle ([Figure 2: see original paper]), suggesting stable ACC2 expression independent of dietary lipid type. Limited research exists on ACC2 mRNA expression in fish fed different lipid sources. Olson et al. found that ACC2 knockout in mouse skeletal muscle did not significantly affect body weight, food intake, or body composition, nor did it alter total malonyl-CoA content or fatty acid oxidation rate, indicating metabolic compensation and minimal impact on energy balance. Alkhateeb et al. reported no direct correlation between skeletal muscle fatty acid oxidation and ACC2 phosphorylation in male rats. Torstensen et al. found that different dietary lipid sources (capelin oil, palm oil, sunflower oil, and mixed oil) did not significantly affect fatty acid -oxidation or growth in Atlantic salmon, corroborating our findings in grass carp muscle. Our previous study also demonstrated that different dietary lipid sources did not significantly affect ACC1 expression in grass carp hepatopancreas.

In this study, hepatopancreas ACC2 mRNA expression increased significantly at 12 hours post-refeeding after starvation, then decreased significantly by 24

hours ([Figure 3: see original paper]). Oh et al. reported significant increases in rat liver ACC2 mRNA expression at 12 and 24 hours post-feeding, indicating transcriptional regulation. Partial differences from our results may reflect species-specific responses. Ryu et al. reported significantly elevated hepatic ACC1 mRNA expression in mice after starvation and refeeding with high-sucrose or high-fat diets. Studies in chickens showed significantly increased ACC1 and fatty acid synthase mRNA expression and decreased CPT-I mRNA expression in liver 2 hours after refeeding. In contrast, grass carp hepatopancreas ACC2 mRNA expression showed no significant difference during 0–6 hours post-refeeding, suggesting fatty acid synthesis predominates during this period.

Conclusion

1. The full-length ACC2 cDNA was successfully cloned from mixed hepatopancreas and heart tissues of grass carp. Key functional sites including ATP-binding and biotin-binding sites are largely conserved compared to other vertebrates.
2. Grass carp ACC2 gene is predominantly expressed in tissues with active lipolysis such as muscle.
3. Different dietary lipid sources do not significantly affect ACC2 mRNA expression in grass carp hepatopancreas.
4. Hepatopancreas ACC2 mRNA expression reaches maximum levels 12 hours after refeeding following starvation.

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