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Elovl4 can effectively elongate C18 polyunsaturated fatty acids in loach *Misgurnus anguillicaudatus*

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ABSTRACT

In this study, full-length cDNA sequences of *elovl4a* and *elovl4b* from loach *Misgurnus anguillicaudatus* were cloned. The full-length cDNAs of loach *elovl4a* and *elovl4b* were 2423 and 2054bp, encoding 315 and 300 amino acids, respectively. The deduced amino acid sequences of *elovl4a* and *elovl4b* in loach both shared the highest identity with those of *Danio rerio*, whereas lower identity score between loach elovl4a and elovl4b was present. Temporal expression and tissue expression of loach *elovl4a* and *elovl4b* were studied by reverse transcriptase PCR. Results of the tissue expression analyses suggested different functions of loach *elovl4a* and *elovl4b*. Functional characterizations of loach *elovl4a* and *elovl4b* on synthesis of fatty acids, especially elongating C18 polyunsaturated fatty acids (PUFAs) to longer-chain fatty acids, were studied by heterologous expression in *Saccharomyces cerevisiae*. Loach elovl4a and elvol4b enzymes were able to elongate all fatty acids tested including 18:2n-6, 18:3n-3, 18:3n-6, 20:4n-6 and 20:5n-3. At last, expression levels of the two *elovl4* genes of loach fin cells incubated with 18:2n-6 and 18:3n-3 of different concentrations were measured. Expressions of *elovl4a* and *elovl4b* of loach fin cells were significantly up-regulated by 18:2n-6 and 18:3n-3. The results obtained here indicated that loach elovl4 could effectively elongate C18 PUFAs. This was a systematic report of elovl4's elongating functions towards C18 and provided an alternative pathway for C20 biosynthesis in fish species.

1. Introduction

Elongases of very long-chain fatty acids (elovl) are key microsomal enzymes involved in the biosynthesis of fatty acids (FA) with C18 or long-chain polyunsaturated fatty acids (LC-PUFAs). Elovl catalyzes the condensation reaction, which is the rate-limiting step in the two carbon elongation of pre-existing fatty acyl chains [1]. The mammalian elovl protein family includes seven members (elovl1-7) and elovl2, elovl4 and elovl5 are considered as key enzymes in the elongation of PUFA [2].

Elovl4 has been cloned from several fish species including zebrafish (*Danio rerio*) [3], Atlantic salmon (*Salmo salar*) [4], Nibe croaker (*Nibea mitsukurii*) [5] and orange-spotted grouper (*Epinephelus coioides*) [6]. Many studies have reported that elovl4 plays a key role in the synthesis of fatty acids and its function is generally considered to elongate C20 fatty acids to longer-chain fatty acids, even up to C36 [3,5]. However, Xie et al. [7] have mentioned that *Scatophagus argus elovl4* can elongate 18:3n-6 to 20:3n-6. So, what is the functional role of elovl4 towards C18 PUFAs in fish?

The loach *Misgurnus anguillicaudatus*, belonging to the family Cobitidae, is one of the most commercially momentous cultured fish species in several Eastern Asian countries including Korea, Japan and China. To unravel functional characterizations of loach *elovl4* genes on synthesis of fatty acids, especially elongating C18 PUFAs to longer-chain fatty acids, we here cloned full-length cDNA sequences of loach *elovl4a* and *elovl4b*, and then investigated their temporal expression and tissue expression. Next, functional characterizations of loach *elovl4a* and *elovl4b* were studied by heterologous expression in *Saccharomyces cerevisiae*. The last but not least, expression levels of the two *elovl4* genes of loach fin cells incubated with 18:2n-6 and 18:3n-3 of different concentrations were measured. This study could enrich knowledges of elovl4's function in elongation of PUFAs, especially towards C18.

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2. Material and methods

2.1. Compliance with ethics guidelines

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Huazhong Agricultural University. All efforts were made to minimize suffering of the loaches.

2.2. Fish

The wild-adult loaches were acquired from Ezhou city of Hubei province in China and reared in a stable temperature (24 ± 1 °C) for about two weeks before tests. The ploidy levels of all loaches were measured by a ploidy analyser (Partec, Germany). Diploid loaches were used here. The loach reproduction and feeding regimes of loach offsprings were based on the methods from our laboratory described by Gao et al. [8].

2.3. Cloning elovl4 genes in loach

Total RNA was isolated from loach liver and RACE cDNA was synthesized according to the methods described in Yan et al. [9]. Two core sequences of loach *elovl4a* and *elovl4b* were obtained from the transcriptome data of our laboratory [10]. The primers for cloning *elovl4a* and *elovl4b* are listed in Table 1 and PCR procedure was running according to manufacture instruction book (Clontceh, USA). The PCR products were collected with 2% agarose (Sangon, China) and cloned into PMD18-T (TaKaRa, Japan). Then the positive transformants were selected and sequenced (Invitrogen, China).

2.4. Sequence and phylogenetic analyses

Similarity analysis of all the sequences was conducted by BLAST program at the National Center of Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of *elovl4a* and *elovl4b* were translated into protein by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The molecular masses and theoretical isoelectric points were predicted by the Compute pI/Mw tool (http://expasy.org/tools/pi_tool.html).

Multiple sequence alignment of *elovl4a* and *elovl4b* were analyzed by Bio-Edit software. The phylogenetic tree of amino acid sequences was established by MEGA 5.05 using the neighbor-joining method (Arizona State University, USA) and aligned sequences were bootstrapped 1000 times by Seqboot, only the bootstrap values higher than 60% were taken into consideration for the consensus tree. All the sequences used here were obtained from NCBI.

2.5. Temporal expression and tissue expression of loach elovl4 genes

Samples of 14 different early life stages (fertilized egg (F), 2 cell (2C), 8 cell (8C), 32 cell (32C), blastula (B), gastrula (G), neurula (N), tail-bud forming (TB), heart-beating (HB), hatching (H), second day after hatching (2nd), tenth day after hatching (10th), twentieth day after hatching (20th), and thirty fifth day after hatching (35th)) and 11 tissues (muscle, kidney, heart, spleen, liver, intestine, gill, brain, testis, ovary and eye) dissected from adult loaches were collected and frozen at −70 °C.

The expressions of loach *elovl4a* and *elovl4b* were studied by reverse transcriptase PCR (RT-PCR). Total RNA was extracted and 5 μg of total RNA of each sample was reverse transcribed into cDNA according to the manufacturer's protocols (TaKaRa, Japan). The procedures of RT-PCR were as follows: initial denaturing at 95 °C for 5min, followed by 35 cycles of denaturation at 95 °C for 30s, annealing at 60 °C for 30s, and extension at 72 °C for 30s. *β-actin* was used as reference gene. Primers used for RT-PCR on cDNA samples are shown in Table 1.

2.6. Functional characterization of loach elovl4 cDNA by heterologous expression in S. cerevisiae

Brain cDNA was used to amplify the PCR fragments corresponding to the ORF of the loach *elovl4a* and *elovl4b*, using TaKaRa Ex Taq® (Takara, Japan). Special primer pairs with restriction sites (underlined in Table 1) of ORF cloning are listed in Table 1. PCR conditions consisted of an initial denaturing step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30s, annealing at 60 °C (*elovl4a*) or 58 °C (*elovl4b*) for 30s, extension at 72 °C for 1min 20s, followed by a final extension at 72 °C for 8 min.

Corresponding restriction endonucleases (Thermo Fisher Scientific, USA) was used to digest the DNA fragments containing loach *elovl4* isoforms and connected to a similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, UK). The purified plasmids containing the putative *elovl4* ORFs were introduced into *S. cerevisiae* competent cells (S.c EasyComp Transformation Kit, Invitrogen). Transformation and selection of yeast with recombinant pYES2-*elovl4* plasmids and yeast culture were performed according to the method described by Agaba et al. [11]. (2004). *S. cerevisiae* minimal medium-uracil was supplied for culture of the recombinant yeast. One of the following FA substrates was added to the medium: linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), γ-linolenic acid (18:3n-6), arachidonic acid (20:4n-6) and eicosapentaenoic acid (20:5n-3). The FA substrates (98–99% pure) were purchased from Cayman Chemical Co. (Ann Arbor, USA). The final concentrations of FA substrates added to the yeast cultures were as following: 0.5 (C18), 0.75 (C20) mM. After 2 days, yeast were harvested and washed for further analyses. As control treatment, yeast transformed with pYES2 containing no insert were cultured under the same condition [3].

2.7. FAME analysis by GC-MS

Total lipids of yeast samples were extracted by homogenisation

Table 1
Nucleotide sequences of the primers for PCR.

Target genes	Primer sequences (5′-3′)
Primers for cDNA ORF cloning	
elovl4a-F	CGGGATCCGATGTTATAAGGCATATAATCAACG
elovl4a-R	GCTCTAGATTATTCACGCTTTGCTCTGCCCTTC
elovl4b-F	GGAATTCGACACCGTCATTCACTTTGTGAATG
elovl4b-R	GCTCTAGATTAATCGCTCTTTGCTCGTTCTTTC
3′ RACE	
elovl4a-3'outer	GGATGCACTGGTGTCTGATCGGATACG
elovl4a-3'inner	ATGAGAGGTTGTTTTCTGTATGGTGTA
elovl4b-3'outer	TTTACCATCTGTGGAGCTGCAAGGGA
elovl4b-3'inner	TGAAAACGGTTTACTTGAGAAGCATTG
5′ RACE	
elovl4a-5'outer	CTACATCTGCCAGCCTGTGGACTACT
elovl4a-5'inner	GGACAGTCATTCTTCGGGGCTCACAT
elovl4b-5'outer	ATGGGTTCCTGGTGGACAGTCGTTT
elovl4b-5'inner	TCTGATTGGTTACGCCGTCACTTTC
Reverse transcriptase PCR (RT-PCR) and real-time quantitative PCR (qPCR)	
elovl4a-F	CCTCACCTTCATCATCCTT
elovl4a-R	CGGCTTCTCATCCATCTT
elovl4b-F	ATGGTGCTGCTTAACTTCTA
elovl4b-R	CGACGCTATCCTGACTTC
β-actin-F	CCGGCCCATCCATCGTCCAC
β-actin-R	CTGCTGCATGGCCAGGTATGGT
GAPDH[a]-F	GACCATGCTGTGCAGAGTCGGATA
GAPDH-R	GGGCTGAAGGGACACTTGGGTAATA

[a] *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.