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Elongation of long-chain fatty acids in rabbitfish *Siganus canaliculatus*: Cloning, functional characterisation and tissue distribution of Elovl5- and Elovl4-like elongases

Óscar Monroig [a,1], Shuqi Wang [b,1], Liang Zhang [b], Cuihong You [b], Douglas R. Tocher [c], Yuanyou Li [b,*]

[a] Instituto de Acuicultura Torre de la Sal (IATS-CSIC), 12595 Ribera de Cabanes, Castellón, Spain
[b] Guangdong Provincial Key Laboratory of Marine Biology, Shantou University, Shantou, Guangdong 515063, China
[c] Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK

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ABSTRACT

Elongases of very long-chain fatty acids (Elovl) catalyse the rate-limiting step of the elongation pathway that results in net 2 C elongation of pre-existing fatty acyl chains. As the biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFA) is particularly relevant in fish, Elovl involved in the pathway have been investigated in various studies. Here we report the molecular cloning, functional characterisation and tissue distribution of two distinct *elovl*-like cDNAs isolated from the herbivorous marine teleost *Siganus canaliculatus*. Unlike the carnivorous marine fish previously investigated, we hypothesise that the rabbitfish has an enhanced LC-PUFA biosynthetic capability as previously anticipated in a former study on fatty acyl desaturases (Fad). The results of the present study showed that rabbitfish expresses at least two *elovl* cDNAs, which have high homology in sequence and function to Elovl5 and Elovl4 elongases that have been investigated previously in other fish species. Furthermore, the results confirm that the activities of the Elovl5 and Elovl4 enzymes enable rabbitfish to perform all the elongation reactions required for the biosynthesis of the physiologically essential $C_{20–22}$ LC-PUFA including eicosapentaenoic (20:5n-3), arachidonic (20:4n-6) and docosahexaenoic (22:6n-3, DHA) acids, as well as the less common very long-chain fatty acids ($>C_{24}$). Rabbitfish is thus the first marine teleost in which genes encoding Fad and Elovl enzymes, with all the activities required for the production of DHA from C_{18} PUFA, have been characterised.

1. Introduction

The molecular and biochemical mechanisms controlling the production of long-chain polyunsaturated fatty acids (LC-PUFA), including eicosapentaenoic (20:5n-3, EPA), docosahexaenoic (22:6n-3, DHA) and arachidonic (20:4n-6, ARA) acids, have been intensively investigated in fish. These studies have been driven by the role that fish play as unique dietary sources of these health-promoting compounds, particularly n-3 LC-PUFA, for human consumers (Bardon et al., 1996; Brouwer et al., 2006; Calder, 2006; Calder and Yaqoob, 2009; Eilander et al., 2007; Ruxton et al., 2007). In addition, a comprehensive understanding of the *de novo* biosynthetic capacity of farmed fish is required to determine which PUFA are the essential fatty acids that must be provided in the diet to ensure normal growth and development (Tocher et al., 2003). Elongases of very long-chain fatty acids (Elovl) are key microsomal enzymes involved in the biosynthesis of fatty acids (FA) with C_{18} or longer chain-lengths. Elovl catalyse the condensation reaction, which is the rate-limiting step in the two carbon elongation of pre-existing fatty acyl chains (Nugteren, 1965). The mammalian Elovl protein family consists of seven members (Elovl 1–7) and, generally, Elovl2, Elovl4 and Elovl5 are regarded as critical enzymes in the elongation of PUFA (Jakobsson et al., 2006).

The zebrafish (*Danio rerio*) Elovl5 was the first Elovl-like cDNA that was cloned and functionally characterised from a fish species (Agaba et al., 2004). Subsequently, further Elovl5-encoding cDNAs were investigated in other species including Atlantic salmon (*Salmo salar*), African catfish (*Clarius gariepinus*), tilapia (*Oreochromis niloticus*), turbot (*Psetta maxima*), gilthead sea bream (*Sparus aurata*), Atlantic cod (*Gadus morhua*), cobia (*Rachycentron canadum*), barramundi (*Lates calcarifer*) and southern (*Thunnus maccoyii*) and northern bluefin (*Thunnus thynnus*) tuna (Agaba et al., 2004, 2005; Gregory et al., 2010; Hastings et al., 2005; Mohd-Yusof et al., 2010; Morais et al., 2009, 2011; Zheng et al., 2009). These studies confirmed that fish Elovl5, similar to mammalian homologues (Jakobsson et al., 2006), have the ability to preferentially elongate C_{18} (18:4n-3 and 18:3n-6) and C_{20} (20:5n-3 and 20:4n-6) PUFA, with only low activity towards C_{22} PUFA (22:5n-3 and 22:4n-6).

Studies on other Elovl enzymes involved in the LC-PUFA biosynthetic pathways have enabled a fuller understanding of the FA elongation pathways in fish. Thus, *elovl2* have been cloned and functionally

* Corresponding author. Tel.: +86 75482903157; fax: +86 75482903473.
E-mail address: yyli@stu.edu.cn (Y. Li).
[1] Joint first authorship.

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characterised from Atlantic salmon (Morais et al., 2009) and zebrafish (Monroig et al., 2009). While activity towards C_{18} PUFA was very low, fish Elovl2 had the ability to elongate C_{20} PUFA, similar to Elovl5, but, in addition, also efficiently elongated the C_{22} substrates, 22:5n-3 and 22:4n-6 (Monroig et al., 2009; Morais et al., 2009). The ability of Elovl2 to elongate 22:5n-3 to 24:5n-3 has been regarded as critical for DHA biosynthesis, as two consecutive elongation steps from 20:5n-3 to 24:5n-3 are required prior to Δ6 desaturation and the peroxisomal chain-shortening steps (Sprecher, 2000). To date, no *elovl2* cDNA has been isolated from a marine fish species, and this had been hypothesised as a factor potentially contributing to their limited ability for DHA biosynthesis (Leaver et al., 2008; Morais et al., 2009).

Recent investigations, however, have suggested that fish Elovl4 exhibit functional similarities to Elovl2, and thus may partly compensate for the apparent absence of Elovl2 in marine species (Monroig et al., 2011a). Specifically, some fish Elovl4 have been demonstrated to effectively elongate C_{20} and C_{22} PUFA, in contrast to mammalian ELOVL4 that appear to operate only towards longer chain (C_{26}) PUFA (Agbaga et al., 2008). Thus, the ability of fish Elovl4 to elongate 22:5n-3 to 24:5n-3 demonstrates that these enzymes have the potential to participate in the production of DHA, similar to Elovl2. Furthermore, similar to mammalian orthologues, teleost Elovl4 have been shown to participate in the biosynthesis of very long-chain fatty acids (VLC-FA) including saturated and polyunsaturated compounds with chain-lengths of $>C_{24}$ (Carmona-Antoñanzas et al., 2011; Monroig et al., 2010a, 2011a). Whereas VLC-FA have key functions in mammalian tissues including skin (Cameron et al., 2007), retina (Aveldaño, 1987, 1988), brain (Robinson et al., 1990) and testis (Furland et al., 2003, 2007a,b), their presence and roles in fish have been barely explored (Poulos, 1995).

Historically, marine fish have been regarded as species with limited capability for *de novo* LC-PUFA biosynthesis in comparison to freshwater and salmonid fish (Tocher, 2010). This view has been supported by a wide variety of evidence including FA compositional analysis obtained from feeding trials, biochemical assays assessing the LC-PUFA biosynthetic ability of primary cell cultures and fish cell lines, and lately through functional characterisation of key enzymes (desaturases and elongases) genes involved in the LC-PUFA biosynthetic pathway (Leaver et al., 2008; Tocher et al., 2003). Compared to freshwater ecosystems, LC-PUFA are readily available in marine environments, and this difference in evolutionary pressure has been hypothesised to account for the apparent loss of some enzymatic activities of the LC-PUFA biosynthetic pathway in marine fish. However, recent studies on the marine teleost rabbitfish have suggested that the above assumption may be too simplistic, as other factors such as trophic level, i.e. the position of an organism in the food chain, might also determine the capacity of a certain species for de novo synthesis of LC-PUFA (Li et al., 2010).

The rabbitfish (*Siganus canaliculatus*), a herbivore consuming algae and seagrasses, occupies a lower trophic level compared to the carnivorous/piscivorous marine finfish upon which the general concept above was forged and, trophically, is more similar to herbivorous freshwater species (Tacon et al., 2010; Woodland, 1990). Here we report on the molecular cloning, functional characterisation and tissue distribution of two Elovl-encoding cDNAs isolated from the rabbitfish. This study aimed to expand our knowledge of the LC-PUFA biosynthesis in rabbitfish, complementing previous studies of other enzymes involved in the pathway, fatty acyl desaturases (Fad) (Li et al., 2008, 2010). Our results on the rabbitfish elongases are discussed within the overall context of LC-PUFA biosynthesis in this species, and the potential impact this could have on the diversification of marine finfish aquaculture to species that have low dependence on dietary LC-PUFA.

2. Materials and methods

2.1. Molecular cloning of rabbitfish elovl5 and elovl4 cDNAs

One μg of total RNA extracted from rabbitfish liver and eye (Trizol reagent, Invitrogen, USA) was reverse transcribed into cDNA using random hexamer primers (Cloned AMV First-Strand cDNA Synthesis Kit, Invitrogen, USA). For *elovl5*, the primers ELO5F (5′-GGTACTACTTCTCCAAGCTCAT-3′) and ELO5R (5′-GTGATGTATCTCTTCCACC-3′) were designed, based on alignment of several fish *elovl5* including those of Atlantic salmon (AY170327), rainbow trout (AY605100), zebrafish (AF532782) and tilapia (AY170326), and they were used to amplify a first fragment of the putative rabbitfish *elovl5* by polymerase chain reaction (PCR) using liver cDNA as template. For *elovl4*, the primers ELO4F (5′-CAGCCTGTCAACTACTCCAATGA-3′) and ELO4R (5′-GTGAGGTATT-

Table 1
Sequences of the primer pairs used and accession numbers of the sequences used as references for primer design in the cloning of the rabbitfish elongase of very long-chain fatty acids (Elovl) ORF and for RT-PCR analysis of gene expression in rabbitfish tissues.

Aim	Transcript	Primer	Primer sequence	Accession no.[a]
Race PCR	*elovl5*	ScE5F 1	5′-TCATGAACTGGATCCCCTGT-3′	GU597350.1
		ScE5F2	5′-GAGACCGTACCTTTGGTGGA-3′	
		ScE5R1	5′-GTTCATGACGAACCACCAGA-3′	
		ScE5R2	5′-GTGTCCATGAACTCGATAAGA-3′	
	elovl4	ScE4F1	5′ -AACCAAGTCAGCTTCCTCCA-3 ′	JF320823.1
		ScE4F2	5′-TATGGTTACTACGGGCTGGC-3′	
		ScE4R1	5′-AGACTGTGTCCAGGAACTCCA-3′	
		ScE4R2	5′-GTAGGAGCTCTTTGGCGATG-3′	
ORF cloning	*elovl5*	ScE5U5F	5′-GGGGGACTTTATGGTGACAA-3′	GU597350.1
		ScE5U3R	5′-TGCGCTACATTGAGAACTGTG-3′	
		ScE5VF	5′-CCCAAGCTTAGGATGGAGGACTTCAATC-3′	
		ScE5VR	5′-CCGCTCGAGTCAATCCACCCTCAGCT-3′	
	elovl4	ScE4U5F	5′-TGTGGAAGCGCTGAGTAGAA-3 ′	JF320823.1
		ScE4U3R	5′-ACTTGCAGGGATGATGAAGC-3′	
		ScE4VF	5′-CCCAAGCTTAGGATGGAGGTTGTAACGC-3′	
		ScE4VR	5′-CCGCTCGAGTTACTCCCTCTTGGCTC-3′	
RT-PCR	*elovl5*	ScE5F2	5′-TTTGGTTTGGAGGCTACCAC-3′	GU597350.1
		ScE5R2	5′-TCCACCAAAGGTACGGTCTC-3′	
	elovl4	ScE4F2	5′-TCCACGTGCTCATGTATGGT-3′	JF320823.1
		ScE4R2	5′-CTTCCTCCTCCACTTTGCTG-3′	
	β-actin	SECTF	5′-CTTCCTTCCTCGGTATGGAGTC-3′	EU107278.1
		ScACTR	5′-AGGTGGAGCAATGATCTT GATC-3′	

[a] GenBank (http://www.ncbi.nlm.nih.gov/).