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Investigating long-chain polyunsaturated fatty acid biosynthesis in teleost fish: Functional characterization of fatty acyl desaturase (Fads2) and Elovl5 elongase in the catadromous species, Japanese eel *Anguilla japonica*

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

[a] Guangdong Provincial Key Laboratory of Marine Biology, Shantou University, Shantou, Guangdong 515063, China
[b] Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling, Scotland FK9 4LA, UK
[c] School of Chinese Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China

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ABSTRACT

The capacity for endogenous production of LC-PUFA from PUFA in euryhaline or diadromous fish is largely unknown other than for Atlantic salmon (*Salmo salar*), an anadromous species, which displays a freshwater pattern. The aim of the present study was to characterize the enzymes of the LC-PUFA pathway in Japanese eel (*Anguilla japonica*), the most important catadromous species currently being farmed. cDNAs of two key genes were cloned and functional assays showed that they encoded a desaturase (Fads2) with Δ6 and Δ8 activity and an elongase (Elovl5) with activity towards C_{18} and C_{20} PUFA, with activities similar to marine fish and an Δ6/Δ8 activity ratio similar to Atlantic salmon. Furthermore, tissue distribution of the mRNA showed a clear marine pattern with the highest expression in the brain and the eyes. Phylogenetic analysis placed the eel cDNAs in line with classical taxonomy. The data suggest that diadromous species display a pattern of LC-PUFA biosynthesis capacity that likely reflects the environmental and nutritional influence of their early life stages rather than those of adult fish. Future studies aim to establish the full range of PUFA desaturases and elongases in Japanese eel and to provide further insight into the importance and relevance of LC-PUFA biosynthesis in fish species and the influence of diadromy.

1. Introduction

Vertebrates, including fish, cannot synthesize polyunsaturated fatty acids (PUFA) *de novo* and so they are essential dietary nutrients (Tocher, 2010). The progressive decline in global fisheries, and increasing importance of farmed fish as the primary dietary source for humans of the beneficial *n*-3 long-chain (LC) PUFA (Tur et al., 2012), has prompted considerable interest in the pathways of endogenous synthesis of LC-PUFA in fish (Tocher, 2003; Turchini et al., 2010).

Dietary PUFA such as linoleic acid (LOA; 18:2*n*-6) and α-linolenic acid (ALA; 18:3*n*-3) can be converted to LC-PUFA in vertebrates, including fish, via a series of desaturation and elongation reactions. The conventionally accepted pathway for the synthesis of arachidonic acid (ARA; 20:4*n*-6) from LOA and eicosapentaenoic acid (EPA; 20:5*n*-3) from ALA requires Δ6 desaturation to 18:3*n*-6/18:4*n*-3 catalyzed by Fads2 fatty acyl desaturase, elongation to 20:3*n*-6/20:4*n*-3 by Elovl5 fatty acyl elongase, and a further Δ5 desaturation catalyzed by Fads1 desaturase (Cook and McMaster, 2004). However, an alternative pathway involving initial elongation of LOA or ALA followed by Δ8 desaturation (an inherent ability of some Fads2 desaturases) may also occur (Monroig et al., 2011a). Docosahexaenoic acid (DHA; 22:6*n*-3) synthesis from EPA can also follow alternative pathways. For many years, the "Sprecher shunt," involving two sequential elongation steps, Δ6 desaturation and limited peroxisomal chain shortening was regarded as the vertebrate pathway (Sprecher, 2000). However, fatty acyl desaturases with Δ4 activity have now been isolated in some teleost fish indicating that the direct route, via elongation to 22:5*n*-3 followed by Δ4 desaturation, is also possible (Li et al., 2010; Morais et al., 2012).

The extent to which any species can convert C_{18} PUFA to LC-PUFA varies, and is associated with their complement of fatty acyl desaturase and elongase genes (Agaba et al., 2004, 2005; Gregory et al., 2010;

* Corresponding author. Tel.: +86 754 86503157; fax: +86 754 86500613.
E-mail address: yyli@stu.edu.cn (Y. Li).
[1] Joint first authorship.

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Hastings et al., 2001, 2005; Mohd-Yusof et al., 2010; Monroig et al., 2009, 2010a,b, 2011a,b, 2012; Morais et al., 2011; Tocher et al., 2006; Zheng et al., 2004, 2005, 2009). It has been generally accepted that freshwater fish species have a greater ability for conversion of C_{18} PUFA to LC-PUFA than marine species (Tocher, 2010), with the limited capacity of marine fish attributed to deficiencies in one or more key enzymes of the endogenous LC-PUFA biosynthesis pathway (Tocher, 2003, 2010). However, this generalization is complicated by the fact that many fish are actually euryhaline or diadromous. Therefore, the euryhaline marine teleost, rabbitfish *Siganus canaliculatus*, can convert C_{18} PUFA to LC-PUFA, and this activity was higher at 10 ppt salinity than that at 32 ppt salinity (Li et al., 2008). Thus, *S. canaliculatus* was the first marine teleost in which genes encoding desaturase and elongase enzymes with all the activities required for the production of DHA from C18 PUFA, had been characterized (Li et al., 2010; Monroig et al., 2012). In contrast, Atlantic salmon (*Salmo salar*), an anadromous species, living in the sea as an adult but returning to freshwater to spawn, displays a freshwater pattern (Tocher, 2003).

Whereas LC-PUFA biosynthesis and anadromy has been extensively studied in Atlantic salmon (Carmona-Antoñanzas et al., 2011; Hastings et al., 2005; Monroig et al., 2010a, 2013; Morais et al., 2009; Zheng et al., 2004, 2005), catadromous species such as anguillid eels have not been studied. The Japanese eel (*Anguilla japonica*) is one such species, spawning in the western North Pacific around the Mariana Ridge with the larvae (leptocephali) carried by the prevailing currents to East Asia where they feed and grow firstly as glass eels and then yellow eels in rivers, lakes and estuaries of Japan, Korea, China, Vietnam and the Philippines (Aida et al., 2003). After several years in freshwater, the eels mature to become silver eels that migrate to the ocean and their spawning grounds (Aida et al., 2003). The Japanese eel is a traditional food fish in East Asia but wild catches are declining and it is now an important farmed species accounting for the major portion of global freshwater eel production of around 260,000 tonnes annually (FAO, 2010).

Understanding the molecular basis of LC-PUFA biosynthesis and regulation in fish will allow the pathway to be optimized to enable efficient and effective use of sustainable plant-based alternatives in aquaculture while maintaining the *n*-3 LC-PUFA content of farmed fish for the human consumer. The specific objectives of the present study were to characterize the genes of LC-PUFA biosynthesis in the catadromous species, Japanese eel, as a key step to understand the mechanisms underpinning variation in the pathway among teleost fish species. In the present paper, we describe the cDNA cloning, functional characterization and tissue distributions of a Fads2 fatty acyl desaturase and Elovl5 PUFA elongase that provide further insight of LC-PUFA biosynthesis in teleost fish species.

2. Materials and methods

2.1. Eel samples

Tissue samples from Japanese eel, *A. japonica*, were obtained from ten adult individuals (body weight 380–400 g) fed on a commercial eel feed and maintained at the facilities of Shantou Manlian Co. Ltd., China. The commercial pellet provided 46% protein and 4.5% lipids, consisted of 66% fish meal but no additional fish oil. The eels were sacrificed after being anaesthetized with an overdose of 3-aminobenzoate methane sulphonate (MS-222; Sigma, China), and tissues including brain, eye, fat (adipose), gill, heart, intestine, kidney, muscle, esophagus and spleen were sampled and immediately frozen in liquid nitrogen, then stored at −70 °C until further use.

2.2. Cloning of putative fads2 and elovl5 from A. japonica

Total RNA was extracted from eel tissues using TRIzol® Reagent (Invitrogen, USA) and first strand cDNA was synthesized using random primers (FastQuant RT Kit, Tiangen Biotech. Co. Ltd., China). The open reading frame (ORF) fragments of the desaturase and elongase cDNAs were isolated by PCR using the primers AJDS1/AJDA1 (desaturase) and AJE5S1/AJE5A1 (elongase), designed on the basis of published sequences of *fads2*-like and *elovl5*-like mRNAs of Japanese eel (GenBank accession EU719615 and EU719614, respectively). PCR was performed using Pfu PCR MasterMix (Tiangen) under the following thermal conditions: initial denaturation at 94 °C for 5 min, 34 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 2 min. The PCR products were cloned into pMD™ 18-T vector (TaKaRa Biotech. Co. Ltd., China). The PCR fragments were sequenced at the DNA Sequencing Service of the Sangon Biotech Co. Ltd. (China). The sequences of all PCR primers used in this study are shown in Table 1.

2.3. Phylogenetic analyses of A. japonica desaturase and elongase

Phylogenetic analysis of the amino acid (aa) sequences deduced from the putative desaturase and elongase cDNAs from Japanese eel and homologous genes from other organisms was performed by constructing a tree using the neighbor-joining method (Saitou and Nei, 1987), with confidence in the resulting tree branch topology measured by bootstrapping through 10,000 iterations. All reference sequences utilized in the phylogenetic analysis are shown in Table 2.

2.4. Functional characterization of A. japonica Fads2 and Elovl5 by heterologous expression in yeast Saccharomyces cerevisiae

A cDNA synthesized with brain, liver and intestine total RNA samples was used as template to amplify the ORFs of *fads2* and *elovl5*, using the Pfu PCR MasterMix (Tiangen). Primers AJDS2/AJDA2 (*fads2*) and AJE5S2/AJE5A2 (*elovl5*) containing restriction enzyme sites (underlined in Table 1) for *Hin*dIII (forward) and *Xba*I (reverse) were used in a PCR consisting of an initial denaturing step at 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. After digestion with the corresponding restriction enzymes, DNA fragments corresponding to the ORFs of *A. japonica fads2* and *elovl5* were ligated into the yeast expression vector pYES2 (Invitrogen, UK). The recombinant plasmids pYES2-fads2 and pYES2-elovl5 were obtained and used to transform yeast *S. cerevisiae* (strain InvSc1) competent cells (S.c. EasyComp Transformation Kit,

Table 1
Primers sequences used for ORF cloning of eel *fads2* and *elovl5* and their tissue expression analysis detected by qRT-PCR.

Primers for ORF cloning of fads2	
AJDS1	5′-CAGGGAGGGAGAATAACGG-3′
AJDA1	5′-CTGAAAATTGTCATAAAGGAAG-3′
AJDS2	5′-CCGAAGCTTGAGCATAAGAGCGATGGG-3′
AJDA2	5′-GGCTCTAGAGGAGGCAGGCTTGAGG-3′
Primers for ORF cloning of elovl5	
AJE5S1	5′-TGGCAGTGGTTCCAAGGTT-3′
AJE5A1	5′-GTGTCAAGACAGCGAGGTTTG-3′
AJE5S2	5′-CCGAAGCTTGATGGACATGGAAATGTT-3′
AJE5A2	5′-GGCTCTAGACTCAGTCTACCCTCAGTT-3′
Primers for real-time quantitative PCR	
fads2	
AJDF3	5′-AGACCCAGCCAGTGGAGTATG-3′
AJDA3	5′-CATTGACCAGACGAGGTCCAC-3′
elovl5	
AJE5F3	5′-TGCTGTGGTCTGGCCTTGTG-3′
AJE5A3	5′-AGCCGTTCTGATGCTCTTTCC-3′
18S	
AJ18SF1	5′-TTAGTGAGGTCCTCGGATCG-3′
AJ18SA1	5′-CCTACGGAAACCTTGTTACG-3′

Note: The accession numbers of nucleotide sequences used for ORF cloning or qPCR of *fads2* and *elovl5* were KJ182968 and KJ182967, respectively. That of 18*S* rRNA was FM946132.