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Biosynthesis of essential fatty acids in *Octopus vulgaris* (Cuvier, 1797): Molecular cloning, functional characterisation and tissue distribution of a fatty acyl elongase

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ABSTRACT

Polyunsaturated fatty acids (PUFAs) have been identified as key nutrients for the common octopus (*Octopus vulgaris*), particularly for its early life-cycle stages (paralarvae). Our overarching aim is to identify the dietary essential fatty acid (FA) for octopus paralarvae through characterisation of the enzymes of endogenous PUFA biosynthetic pathways. Here we report on the molecular cloning and functional characterisation of a cDNA encoding a putative elongase of very long-chain fatty acids (Elovl), a critical enzyme that catalyses the elongation of FA including PUFA. Our results suggest that the octopus Elovl is phylogenetically related to Elovl5 and Elovl2, two elongases with demonstrated roles in PUFA biosynthesis in vertebrates. Further evidence supporting a role of the octopus Elovl in PUFA biosynthesis was provided through functional characterisation of its activity in yeast. It was confirmed that expression of the octopus Elovl conferred on yeast the ability to elongate some C18 and C20 PUFAs, while C22 PUFA substrates remained unmodified. Therefore, the substrate specificities exhibited by the octopus elongase were consistent with those of vertebrate Elovl5. Interestingly, the octopus Elovl elongated n−6 PUFA substrates more efficiently than their homologous n−3 substrates, suggesting that n−6 PUFA may have particular biological significance in *O. vulgaris*. Finally, we investigated the potential role of the newly cloned Elovl in the biosynthesis of non-methylene-interrupted FA, compounds typically found in marine invertebrates and confirmed to be also present in the common octopus.

1. Introduction

Cephalopods have emerged as prime candidates for diversifying aquaculture. Among the species studied, the common octopus (*Octopus vulgaris*, Cuvier, 1797) has received special attention and relevant aspects of its culture such as husbandry (Estefanell et al., 2012; Iglesias et al., 2006), behaviour (Di Cristo et al., 2005; Valverde and Garcia, 2005), reproduction (Estefanell et al., 2010; Otero et al., 2007; Wodinsky, 2008), pathologies (Castellanos-Martinez and Gestal, 2011) and nutrition (Estefanell et al., 2011; Fuentes et al., 2011; Navarro and Villanueva, 2000, 2003; Quintana, 2009; Seixas et al., 2010; Viciano et al., 2011; Villanueva, 1994; Villanueva and Bustamante, 2006; Villanueva et al., 2004, 2009) have been studied. Despite considerable effort, the production of the common octopus in captivity is limited to on-growing wild-captured specimens in floating cages (Iglesias et al., 2007), as the octopus life cycle has not yet been closed at commercial scale. While limited success in the production of juvenile octopuses has been achieved (Iglesias et al., 2002, 2004; Villanueva, 1995), the massive mortalities occurring during early life-cycle stages (paralarvae) have become an, as yet, unresolved zootechnical issue that requires further investigation.

Polyunsaturated fatty acids (PUFAs), in particular docosahexaenoic acid (DHA, 22:6n−3) and eicosapentaenoic acid (EPA, 20:5n−3), have been previously suggested as critical dietary components for octopus paralarvae (Navarro and Villanueva, 2003). We have recently initiated a series of studies to identify the dietary essential fatty acids (FA) for octopus paralarvae. Due to the obvious difficulties in conducting feeding trials with octopus paralarvae, our approach is to characterise the enzymes involved in PUFA biosynthesis as they dictate the ability of a certain species to endogenously produce PUFA (Bell and Tocher, 2009). These studies will help us to design balanced diets for octopus paralarvae that do not compromise their endogenous capabilities for PUFA biosynthesis.

Previously, we reported the molecular cloning and functional characterisation of a fatty acyl desaturase (Fad) from *O. vulgaris* (Monroig et al., 2012a). The substrate specificity of the octopus Fad revealed that this enzyme was a Δ5-like Fad and thus we provided for the first time molecular evidence of such an enzymatic activity in molluscs (Monroig et al., 2012a). Interestingly, the Δ5 Fad potentially enables the common octopus to endogenously convert 20:4n−3 and 20:3n−

Abbreviations: ARA, arachidonic acid; BHT, butylated hydroxy toluene; DHA, docosahexaenoic acid; Elovl, elongase of very long-chain fatty acids; EPA, eicosapentaenoic acid; EST, expressed sequence tag; FA, fatty acid; Fad, fatty acyl desaturase; FAME, fatty acid methyl ester; GC-MS, gas chromatography–mass spectrometry; NMI FA, non-methylene interrupted fatty acid; ORF, open reading frame; OD, optical density; PCR, polymerase chain reaction; PUFA, polyunsaturated fatty acid.

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6 to 20:5n−3 (EPA) and arachidonic acid (ARA, 20:4n−6), respectively. The latter are regarded as critical PUFA in a variety of physiological processes ensuring normal cellular function (Funk, 2001). Rather than a role in the biosynthesis of EPA, we hypothesised that the Δ5 Fad activity may actually contribute to the endogenous biosynthesis of ARA in the octopus, as high concentrations of ARA encountered in adult octopus tissues were unlikely to be exclusively of dietary origin. In addition to the potential participation of the octopus Δ5 Fad in ARA biosynthesis, the common octopus Δ5 Fad might also have a role in the biosynthesis of non-methylene interrupted fatty acids (NMI FA), compounds with unusual unsaturation features that have been found in a variety of marine invertebrates (Barnathan, 2009; Kornprobst and Barnathan, 2010).

The biosynthesis of PUFA including NMI FA in marine molluscs has been investigated previously (De Moreno et al., 1976; Waldock and Holland, 1984; Zhukova, 1986, 1991, 2007). The PUFA biosynthetic capability of molluscs seems to vary among species according to enzymatic activities present in each species. However, it has been shown that some molluscs have active PUFA biosynthetic pathways and, in addition to the above mentioned Δ5 desaturase, active FA elongation systems also appear to be present. Using radioactive FA, De Moreno et al. (1976) were able to show that the clam *Mesoderma mactroides* could elongate both 18:3n−3 and 18:2n−6. Later, Waldock and Holland (1984) demonstrated that the Pacific oyster *Crassostrea gigas* had the ability to desaturate and elongate ^{14}C-labelled PUFA precursors provided through the diet (microalgae) to PUFA including 20:5n−3 and 22:6n−3. Investigations with ^{14}C substrates demonstrated that other molluscs such as *Scapharca broughtoni*, *Callista brevisiphonata* and *Mytilus edulis* can biosynthesise the NMI Δ7,13 22:2 and Δ7,15 22:2 by elongation from Δ5,11 20:2 and Δ5,13 20:2, respectively (Zhukova, 1986, 1991). In addition to biochemical assays with radiotracers, indirect evidence of FA elongase activity in molluscs was provided analytically (Joseph, 1982). For instance, the unusual NMI FA Δ5,9,15 24:3 and Δ5,9,17 24:3 found in the limpets *Cellana grata* and *Collisella dorsuosa* were suggested to derive from the typical NMI FA Δ7,13 22:2 and Δ7,15 22:2, respectively, by chain elongation and subsequent Δ5 desaturation (Kawashima, 2005).

In vertebrates, elongase of very long-chain fatty acid (Elovl) enzymes catalyse the addition of 2 carbons to a preexisting fatty acyl chains (Jakobsson et al., 2006). There are seven distinct member of the ELOVL protein family in vertebrates (designated Elovl 1–7) and many of them have been functionally characterised (see reviews by Guillou et al., 2010; Jakobsson et al., 2006; Monroig et al., 2011a). In contrast, studies of Elovl genes and proteins from non-vertebrate organisms are scarce, with only a few examples such as elongases from the nematode *Caenorhabditis elegans* (Beaudoin et al., 2000) and the marine protist *Thraustochytrium* sp. (Heinz, 2001; Jiang et al., 2008). To date, no elongases from molluscs have been reported.

As a further step towards understanding the EFA requirements of common octopus, the present study reports the molecular cloning, functional characterisation and tissue distribution of transcripts encoding a putative elongase involved in PUFA biosynthesis. In order to investigate a potential role of the newly cloned elongase in NMI FA biosynthesis in the common octopus, we also analysed the double bond features of NMI FA found in specific tissues of octopus adult specimens.

2. Materials and methods

2.1. Tissue samples

Tissue samples from common octopus were obtained from the dissection of two (male and female) adult individuals (~1.5 kg) captured by artisanal fisheries along the Mediterranean East coast of Spain. The octopuses were anesthetised by inmersion in seawater at 4 °C and sacrificed by direct brain puncture and tissues including nerve, nephridium, hepatopancreas, brain, caecum, gill, muscle, heart and gonad were sampled and immediately frozen at −80 °C until further analysis.

2.2. Fatty acyl elongase cDNA cloning

Total RNA was extracted from octopus tissues using TRIzol® (Gibco BRL, Grand Island, NY, USA) reagent following manufacturer's instructions. Subsequently, first strand cDNA was synthesised from 1 μg total RNA using a Verso™ cDNA kit (ABgene, Rockford, IL, USA) primed with random hexamers. In order to amplify the first fragment of the elongase cDNA, the amino acid (aa) sequences of Elovl5 proteins from *Homo sapiens* (NP_068586.1), *Rattus norvegicus* (NP_599209.1), *Bos taurus* (NP_001040062.1), *Danio rerio* (NP_956747.1) and *Pagrus major* (ADQ27303.1) were aligned using BioEdit v5.0.6 (Tom Hall, Department of Microbiology, North Carolina State University, USA). Conserved regions were used for in silico searches of mollusc expressed sequence tags (EST) using NCBI tblastn tool (http://www.ncbi.nlm.nih.gov/). Several ESTs displaying high similarity with Elovl encoding genes were identified from the molluscs *Mytilus galloprovinciallis* (gb|FL495089.1| and gb|FL499406.1|), *Euprymna scolopes* (gb|DW256301.1|), and *Lymnaea stagnalis* (gb|FC701557.1|, gb|FC773093.1|, gb|FC770692.1| and gb|FC696214.1|). Additionally, a search of the owl limpet *Lottia gigantea* genome was performed using the zebrafish Elovl5 (NP_956747.1) sequence with the tblastn tool at http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html. After processing, the mollusc Elovl-like sequences were aligned (Bioedit) for the design of the primers UNIEloF (5′-TTGTGGTGGTATTACTTCTC-3′) and UNIEloR (5′-GTAATATACTTTTTCCACCA-3′) that were used for polymerase chain reaction (PCR) using GoTaq® Green Master Mix (Promega, Southampton, UK), and using a mixture of cDNA from gonads, brain, nerve and caecum as template. The PCR cycling conditions consisted of an initial denaturing step at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The PCR fragment was sequenced at the DNA Sequencing Service of the IBMCP-UPV (Valencia, Spain) and gene-specific primers were designed for 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR (FirstChoice® RLM-RACE kit, Ambion, Applied Biosystems, Warrington, UK) to produce a full-length cDNA. Details of all primers used for RACE PCR are given in Table 1.

For 5′RACE PCR, a positive fragment was obtained by nested PCR approach. The first round PCR was performed using the adapter-specific 5′RACE OUTER primer and the gene-specific reverse primer OVEloR1, with an initial denaturing step at 95 °C for 2 min, followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 75 s, followed by a final extension at 72 °C for 5 min (GoTaq® Colorless Master Mix, Promega). First round PCR products were used as template for nested PCR with primers 5′RACE INNER and OVEloR2 in a 32-cycle reaction under the same thermal conditions as above. For 3′RACE PCR, a similar nested approach was followed with first round PCR performed with primers OVEloF1 and 3′RACE OUTER, with an initial denaturating step at 95 °C for 1 min, followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 2 min, followed by a final extension at 72 °C for 5 min (GoTaq® Colorless Master Mix, Promega). First round PCR products were then used as template for nested PCR with primers OVEloF2 and 3′RACE INNER, with thermal conditions as above. RACE PCR products were cloned into pGEM-T Easy Vector (Promega) and sequenced as above.

2.3. Sequence and phylogenetic analyses

Using ClustalW (Bioedit), the deduced aa sequence of the newly cloned *O. vulgaris* elongase cDNA was aligned with that of a predicted elongase found in the gastropod owl limpet (termed '*L. gigantea* Elovl transcript 1', jgi|Lotgi1|224291|), as well as those of protein homologues including the human ELOVL5 (gb|NP_068586|) and ELOVL2 (gb|NP_060240|), and the zebrafish Elovl5 (gb|NP_956747|) and Elovl2 (gb|NP_001035452|). The aa sequence identity between Elovl-like