Comparative Biochemistry and Physiology, Part B 203 (2017) 84–91

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Molecular and functional characterization of a *fads2* orthologue in the Amazonian teleost, *Arapaima gigas*

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ARTICLE INFO

Article history:
Received 8 June 2016
Received in revised form 8 September 2016
Accepted 27 September 2016
Available online 29 September 2016

Keywords:
Arapaima gigas
Fatty acid desaturase (Fads)
Long-chain polyunsaturated fatty acids (LC-PUFAs)
Teleosts
Evolution

ABSTRACT

The Brazilian teleost *Arapaima gigas* is an iconic species of the Amazon. In recent years a significant effort has been put into the farming of arapaima to mitigate overfishing threats. However, little is known regarding the nutritional requirements of *A. gigas* in particular those for essential fatty acids including the long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The ability to biosynthesize LC-PUFA is dependent upon the gene repertoire of fatty acyl desaturases (Fads) and elongases (Elovl), as well as their fatty acid specificities. In the present study we characterized both molecularly and functionally an orthologue of the desaturase fatty acid desaturase 2 (*fads2*) from *A. gigas*. The isolated sequence displayed the typical desaturase features, a cytochrome b_5-domain with the heme-binding motif, two transmembrane domains and three histidine-rich regions. Functional characterization of *A. gigas fads2* showed that, similar to other teleosts, the *A. gigas fads2* exhibited a predominant Δ6 activity complemented with some capacity for Δ8 desaturation. Given that *A. gigas* belongs to one of the oldest teleostei lineages, the Osteoglossomorpha, these findings offer a significant insight into the evolution LC-PUFA biosynthesis in teleosts.

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) play vital roles in numerous biological processes. They participate in structural functions as major components of biomembranes and are also involved in processes such as the inflammatory response, reproduction (Wall et al., 2010; Robinson and Mazurak, 2013), neural development (Perica and Delaš, 2011) and can have beneficial effects in pathological conditions such as cardiovascular disease (Psota et al., 2006; Jump et al., 2012). LC-PUFA are often defined as compounds with 20 to 24 carbon atoms and three or more double bonds (unsaturations), and can be classified into two main groups: the omega-6 (ω6 or n-6) and the omega-3 (ω3 or n-3) LC-PUFA, based upon the position of the first double bond in relation to the methyl end carbon (CH_3) (Monroig et al., 2011a). LC-PUFA of the n-6 and n-3 series can be of dietary origin or, alternatively, they can be biosynthesized from dietary essential fatty acids (EFA) such as linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), respectively, through a series of sequential biochemical reactions, mediated by elongation of very long-chain fatty acid protein (Elovl) and fatty acyl desaturases (Fads).

The ability to endogenously synthesize LC-PUFA from dietary fatty acids (FA) differs markedly among vertebrate species (Rivers et al., 1975; Bauer, 1997; Tocher, 2003; Burdge and Calder, 2005; Fonseca-Madrigal et al., 2014; Castro et al., 2016; Monroig et al., 2016a, 2016b). This variation may be primarily attributed to differences in the *elovl* and *fads* gene repertoire, as well as their associated fatty acid substrate specificities. For instance, mammals have several *FADS* genes of which *FADS1* encodes a Δ5 desaturase and *FADS2* encodes a desaturase with Δ6 preference, in addition to Δ4 activity reported in some mammals (Park et al., 2009, 2015). In contrast, teleost fish examined to date have been found to possess exclusively *FADS2* orthologues (Castro et al., 2012, 2016). However, while mammalian FADS enzymes are essentially mono-functional, mechanisms of bifunctionalization (i.e., acquisition of additional/alternative substrate specificities) have been described in several teleost Fads2. Thus, Fads2 with dual Δ6Δ5 desaturase activities have been described in *Danio rerio* (Hastings et al., 2001), *Siganus canaliculatus* (Li et al., 2010), *Oreochromis niloticus* (Tanomman et al., 2013), *Chirostoma estor* (Fonseca-Madrigal et al., 2014) and *Clarias gariepinus* (Oboh et al., 2016). In addition,

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http://dx.doi.org/10.1016/j.cbpb.2016.09.007
1096-4959/

S. canaliculatus and *C. estor* possess a duplicated *Fads2* that exhibit Δ4 desaturase activity (Li et al., 2010; Fonseca-Madrigal et al., 2014), a type of enzyme also found in *Solea senegalensis* (Morais et al., 2012) and *Channa striata* (Kuah et al., 2015). Moreover, in agreement with the abilities reported in the baboon Δ6-desaturase (Park et al., 2009), the majority of teleost Fads2 desaturases have been demonstrated to possess the capability for Δ8 desaturation (Monroig et al., 2011b). Overall the complement of LC-PUFA biosynthetic enzymes, namely FADS and ELOVL, as well as their functionalities, dictates the ability of a species for the conversion of C_{18} PUFA (LA and ALA) into physiologically important LC-PUFA including arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Bell and Tocher, 2009; Castro et al., 2016). Importantly, the investigation of Fads and Elovl in fish has primarily focused on farmed species since both Fads and Elovl capabilities underpin the efficiency of these fish species to utilize the C_{18} PUFA present in vegetable oils (VO) currently used as sustainable replacements for dietary fish oils (FO) in aquafeeds (Tocher, 2010). Therefore a clear understanding of LC-PUFA biosynthesis pathways is critical to understand the potential limitations of farmed fish species and for the implementation of dietary strategies to fulfil essential requirements and ensure normal growth and development in captivity.

An iconic species of the Amazon, so-called "pirarucú" (*Arapaima gigas*), is one of the largest freshwater and air-breathing fishes in the world, and has been extensively fished since the 18th century (Veríssimo, 1895; Goulding, 1980). In the early 1970's over-exploitation of *A. gigas* led to its near extinction (Goulding, 1980) and listing in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). To overcome this threat, considerable effort has been put into developing the sustainable farming of this species. However, despite some important advances, critical knowledge in key areas such as physiology and nutrition is still scarce in this species. Much of the published research on *A. gigas* has focused on the understanding and evolution of the air-breathing capacity (Brauner et al., 2004; Gonzalez et al., 2010), general health and aquaculture practices (Ribeiro et al., 2011; Bezerra et al., 2014) and, more recently, the potential use of *A. gigas* scales as biomaterials (Torres et al., 2015). In contrast, few studies have addressed the dietary requirements of *A. gigas* (Ituassú et al., 2005; Andrade et al., 2007; Ribeiro et al., 2011), stressing the need for a broader understanding of the metabolism of this carnivorous species. Here, we describe the isolation and functional characterization of a cDNA from *A. gigas* orthologous to *fads2* desaturases, key enzymes in LC-PUFA biosynthetic pathways and crucial elements in determining EFA requirements in this species. The phylogenetic position of *A. gigas* within one of the most ancient teleost lineages, the Osteoglossomorpha, brings new insights into the evolution of the LC-PUFA biosynthesis cascade in both fish and vertebrates in general.

2. Materials and methods

2.1. Molecular cloning of the A. gigas fads gene

Total RNA was extracted from a range of *A. gigas* tissues using the Illustra RNAspin Mini kit (GE Healthcare, UK). The RNA extraction process included an on-column DNase I treatment (provided in the kit). RNA integrity was assessed on a 1% agarose TAE gel stained with GelRed™ nucleic acid stain (Biotium, Hayward, CA, USA). The Quant-iT™ RiboGreen® RNA Assay Kit (Life Technologies, Carlsbad, CA, USA) was used to measure total RNA concentration. Reverse transcription reactions were performed with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA).

Arapaima gigas FADS gene was isolated in three main steps. First, degenerate primers targeting the Fads gene were designed using CODEHOP (Rose et al., 2003) available at http://blocks.fhcrc.org/codehop.html. The initial polymerase chain reaction (PCR) was performed with a degenerate primer set and Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), set for a final volume of 20 μl, with 500 nM of sense and antisense primers, and 1 μl of *A. gigas* cDNA pool (see Table 1 for primers, PCR conditions). In the second step, the partial *fads* sequence was further extended by Rapid amplification of cDNA ends (RACE) PCR using as template 5′ and 3′ RACE ready cDNA prepared with SMARTer™ RACE cDNA Amplification Kit (Clontech, CA, USA). Gene specific primers for RACE were designed using the previously isolated fragment and RACE PCR was performed with Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) using 1 μl of gene specific primer combined with 2 μl Universal primer mix (Clontech) (see Table 1 for primers and PCR conditions). The resulting 5′and 3′sequences were assembled to produce the full open reading frame (ORF) *fads*-like cDNA. In the final step, the full ORF of *A. gigas* FADS was isolated using 1 μl of *A. gigas* cDNA pool, and Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), set for a final volume of 20 μl, with 500 nM of sense and antisense primers (see Table 1 for primers and PCR conditions). In each step resulting PCR products were analyzed in 1% agarose gel, purified with NZYGelpure (NZYTech, Lisbon, Portugal) and confirmed by sequencing (GATC Biotech Constance, Germany). The final, full ORF sequence was translated and submitted to pFAM and NCBI for blastp searches retrieving Fads-like profile (Accession number: KX809739).

2.2. Sequence collection, phylogenetic and 2D structural analysis

Fads amino acid (aa) sequences were retrieved from Genbank and Ensembl (for accession numbers see Table 2). Sequences were aligned with MAFFT using the L-INS-i method (Katoh and Toh, 2008). The sequence alignment was stripped from all columns containing gaps leaving 374 gap-free sites for phylogenetic analysis. Maximum likelihood phylogenetic analysis was performed in PhyML v3.0 server (Guindon et al., 2010) using smart model selection resulting in LG + G + I + F, and branch support was calculated using 1000 bootstraps. Using the same alignment a second Bayesian phylogenetic analysis was performed using MrBayes v3.2.3 available in CIPRES Science Gateway V3.3 (Miller et al., 2015). MrBayes was run for 1 million generations with the following parameters: rate matrix for aa = mixed, nruns = 2, nchains = 4, temp = 0.2, sampling set to 500 and burin to 0.25. The resulting trees were visualized in Fig Tree V1.3.1 available at http://tree.bio.ed.ac.uk/software/figtree/ and rooted at mid-point. *A. gigas* aa sequence was submitted to TOPCONS web server for prediction of 2D topology, with all parameters set to default (http://topcons.net/) (Tsirigos et al., 2015), and results visualized using Potter web application (http://wlab.ethz.ch/protter) (Omasits et al., 2014).

2.3. Yeast expression assays and fatty acid analysis

The *A. gigas fads* ORF was isolated with two sequential PCR with Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA) as described above. The first PCR was performed with an *A. gigas* cDNA pool and primers (AgigasFADS_ORF_F and AgigasFADS_ORF_R, Table 1) targeting the full ORF. The PCR product was diluted (1:50) and used as template for the second PCR performed with primers containing restriction sites for *Kpn*I (AgigasFADS_pYES_KpnI_F) and *Xba*I (AgigasFADS_pYES_XbaI_R) (Table 1). The final PCR product was purified and digested with the appropriate restriction enzymes and cloned into the yeast expression vector pYES2 (Invitrogen, CA, USA). Transformation and culture of yeast *Saccharomyces cerevisiae* were conducted as previously described (Hastings et al., 2001; Agaba et al., 2004; Oboh et al., 2016). Briefly, transgenic yeast expressing the *A. gigas fads* ORF were grown in the presence of PUFA including Δ6 (18:3n-3 and 18:2n-6), Δ8 (20:2n-6 and 20:3n-3), Δ5 (20:4n-3; 20:3n-6) and Δ4 (22:5n-3 and 22:4n-6) desaturase substrates. PUFA substrates, added as sodium salts, were supplemented in the yeast medium at final concentrations of 0.5 mM (C_{18}), 0.75 mM (C_{20}) and 1.0 mM (C_{22}) as uptake efficiency decreases with increasing chain