Aquaculture 488 (2018) 199–206

Contents lists available at ScienceDirect

Aquaculture

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

Essential fatty acid metabolism and requirements of the cleaner fish, ballan wrasse *Labrus bergylta*: Defining pathways of long-chain polyunsaturated fatty acid biosynthesis

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ARTICLE INFO

Keywords:
Essential fatty acids
Ballan wrasse
Fatty acyl desaturase
Elongation of very long chain fatty acid protein

ABSTRACT

Ballan wrasse (*Labrus bergylta*) is an effective counter-measure against sea lice used by Atlantic salmon farmers, proving to be more effective and economical than drugs or chemical treatments alone. There are currently efforts underway to establish a robust culture system for this species, however, essential fatty acid dietary requirements are not known for ballan wrasse. In the present study, we isolated and functionally characterised ballan wrasse fatty acid desaturase (Fads) and elongation of very long-chain fatty acids (Elovl) protein to elucidate their long-chain polyunsaturated fatty acid (LC-PUFA) biosynthetic capability. Sequence and phylogenetic analysis demonstrated that the cloned genes were *fads2* and *elovl5* orthologues of other teleost species. Functional characterisations of *fads2* and *elovl5* were performed using the yeast (*Saccharomyces cerevisiae*) heterologous expression system. The Fads2 showed Δ6 desaturase activity towards 18:3n–3, 18:2n–6 and 24:5n–3, and Δ8 desaturase activity towards 20:3n–6 and 20:2n–6. The Elovl5 showed elongase activities towards various C_{18} and C_{20} fatty acids. Therefore, 20:4n–3 and 20:3n–6 can be synthesised from 18:3n–3 and 18:2n–6, respectively in ballan wrasse *via* two possible pathways, the Δ6 (Δ6 desaturation – elongation) and Δ8 (elongation – Δ8 desaturation) pathways. However, due to the absence of Δ5 desaturase activity and no other Fads2 in their genome, 20:5n–3 (eicosapentaenoic acid, EPA) and 20:4n–6 (arachidonic acid, ARA) cannot be synthesised from C_{18} PUFA precursors and they could consequently be regarded as dietary essential fatty acids for ballan wrasse. Since no Δ4 desaturase activity was detected in ballan wrasse Fads2, 22:6n–3 (docosahexaenoic acid, DHA) can only be synthesised from EPA *via* the Sprecher pathway comprising two sequential elongation steps to produce 24:5n–3 followed by Δ6 desaturation and chain shortening. Although ballan wrasse Elovl5 had no elongase activity towards C_{22}, other elongases such as Elovl4 exist in the ballan wrasse genome that may be able to produce 24:5n–3. Therefore, as ballan wrasse Fads2 can desaturate 24:5n–3 to produce 24:6n-3, it can be assumed that ballan wrasse can synthesise DHA from EPA.

1. Introduction

Plant ingredients are commonly used nowadays in aquafeeds to replace marine ingredients including fishmeal and fish oil (FO) (Turchini et al., 2010). While this strategy has numerous advantages associated with lower cost, high availability and perceived environmental sustainability (Turchini et al., 2010), a major drawback derived from use of plant ingredients is related to their poorer nutritional profile in comparison to marine ingredients. With regards to the oil component of the diet, plant ingredients, unlike marine ingredients, completely lack long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) including eicosapentaenoic acid (EPA; 20:5n–3), docosahexaenoic acid (DHA; 22:6n–3) and arachidonic acid (ARA; 20:4n–6), physiologically critical compounds necessary for normal cellular function of all vertebrates including fish (Tocher, 2010, 2015). High inclusion of plant ingredients, particularly vegetable oils (VO), thus results in decreased accumulation of LC-PUFA such as EPA and DHA (Henriques et al., 2014; Sprague et al., 2016), which compromises the nutritional quality of farmed fish products for human consumers (Swanson et al., 2012; Calder, 2015). Further detrimental effects from VO use in aquafeed include those on the health of fish themselves, at least partly related to potentially limited provision of essential fatty acids (EFA) such as EPA, DHA and ARA in the diet (Tocher, 2015). While endogenous production (biosynthesis) of LC-PUFA from α-

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https://doi.org/10.1016/j.aquaculture.2018.01.039
Received 4 December 2017; Received in revised form 23 January 2018; Accepted 24 January 2018

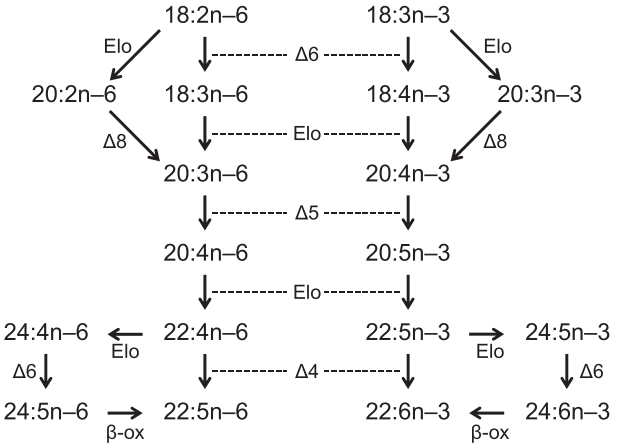

Fig. 1. The biosynthetic pathways of LC-PUFA in vertebrates. "Δx" refers to desaturation activities by Fads enzymes, whereas "Elo" are reactions catalysed by Elovl enzymes. B-ox, beta-oxidation.

linolenic acid (ALA; 18:3n–3) and linoleic acid (LA; 18:2n–6), present in a range of VO sources, is possible in some fish species, EFA requirements in marine species typically cannot be satisfied by C_{18} polyunsaturated fatty acids (PUFA), ALA and LA, as they have limited LC-PUFA biosynthetic capability (Tocher, 2010; Castro et al., 2016). With diversification of finfish aquaculture often involving marine species, it is important to understand LC-PUFA biosynthesis capabilities so that nutritionally balanced diets including plant ingredients can be designed without compromising the EFA requirements and health of marine fish species.

Studies aiming to elucidate LC-PUFA biosynthesis in a large variety of fish species have enabled us to establish that the LC-PUFA biosynthetic capacity of fish varies among species depending upon the repertoire and function of two types of enzymes, namely fatty acyl desaturases (Fads) and elongation of very long chain fatty acids (Elovl) proteins (Fig. 1). All Fads enzymes characterised from teleosts, the fish group to which virtually all species farmed belong, are Fads2 (Castro et al., 2012; Kabeya et al., 2017). In agreement with mammalian FADS2, the majority of teleost Fads2 have Δ6 desaturase activity and thus can operate towards ALA and LA to produce 18:4n–3 and 18:3n–6, respectively (Fig. 1) (Castro et al., 2016; Kabeya et al., 2017). Recently it has been demonstrated that most teleost Fads2 can also catalyse the Δ6 desaturation that converts 24:5n–3 to 24:6n–3, a critical reaction for DHA biosynthesis *via* the so-called "Sprecher pathway" (Fig. 1) (Oboh et al., 2017a). In some species, Δ6 Fads2 have evolved by acquiring either further desaturase capacities to become bifunctional Δ6Δ5 desaturases (subfunctionalisation) or new activities like monofunctional Δ5 and Δ4 desaturases (Castro et al., 2016; Kabeya et al., 2017). With the exception of Δ4 desaturases, fish Fads2 exhibit Δ8 desaturase capability, which appears to be an inherent feature among vertebrate Fads2 enzymes (Monroig et al., 2011; Park et al., 2009).

Along with Fads, LC-PUFA biosynthesis requires the action of Elovl (or elongase) enzymes (Fig. 1). Three types of Elovl, namely Elovl5, Elovl2 and Elovl4, play major roles in LC-PUFA biosynthesis (Castro et al., 2016) and differ from each other according to their substrate specificity (Jakobsson et al., 2006). Elovl5 is found in virtually all teleosts and has preference for C_{18} and C_{20} PUFA, but not C_{22} substrates (Castro et al., 2016). In agreement with a shared evolutionary origin (Monroig et al., 2016), Elovl2 can elongate C_{20} PUFA like Elovl5 but, in contrast, Elovl2 has the ability to elongate C_{22} PUFA substrates and only limited activity towards C_{18} PUFA (Castro et al., 2016). Importantly, the *elovl2* gene has been lost during the evolution of teleosts and, as a consequence, the vast majority of marine fish species currently farmed do not possess this type of elongase (Castro et al., 2016). It has been postulated that loss of elongation capacity towards C_{22} PUFA through the absence of Elovl2 in marine fish can be partly compensated by another elongase, Elovl4 (Monroig et al., 2010, 2011) since, in addition to the biosynthesis of very long-chain ($> C_{24}$) PUFA (Oboh et al., 2017b), fish Elovl4 can elongate C_{22} PUFA (Monroig et al., 2010; Jin et al., 2017). The above mentioned diversity of gene complement and substrate specificities in fish LC-PUFA biosynthesising enzymes demonstrates that species-specific studies on Fads and Elovl are required to unequivocally elucidate the capacity to utilise dietary VO of new fish species that are candidates for aquaculture diversification.

Ballan wrasse *Labrus bergylta* has been used in the biological control of the ectoparasite *Lepeophtheirus salmonis* in salmon aquaculture (Bjordal, 1991; Torrissen et al., 2013; Aaen et al., 2015). As the demand for ballan wrasse juveniles grows, husbandry practices including feeding and nutrition (Hamre et al., 2013) for this species are rapidly developing in order to guarantee supply and avoid exploitation of wild populations. With regards to essential lipids, it has been reported that higher dietary DHA:EPA ratio is positively correlated with growth performance of ballan wrasse (Kousoulaki et al., 2015), but little is known about their capacity to utilise dietary VO to meet EFA requirements. For that purpose, the present study aimed to clone and functionally characterise *fads2*- and *elovl5*-like cDNAs with putative roles in the LC-PUFA biosynthetic pathways of ballan wrasse.

2. Materials and methods

2.1. Sample collection, RNA extraction and cDNA synthesis

All experiments were subjected to ethical reviewed and approved by the University of Stirling through the Animal and Welfare Ethical Review Body. The project was conducted under the UK Home Office in accordance with the amended Animals Scientific Procedures Act implementing EU Directive 2010/63. One single ballan wrasse juvenile (42.6 g) was obtained from Otter Ferry Seafish Ltd. (Otter Ferry, Scotland, UK) and humanely euthanised with an overdose of anaesthetic (metacaine sulphonate, MS-222; PHARMAQ, UK) prior to dissection. Tissues including brain, liver, intestine and eye were chosen for RNA extraction based on their roles in LC-PUFA biosynthesis (Hamid et al., 2016). Total RNA from each tissue sample (~30 mg) was extracted by TRIzol® (Thermo Fisher Scientific, UK) following the manufacturer's instructions. Subsequently, cDNA was synthesised from 2 μg of total RNA with a random primer using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

2.2. Molecular cloning of L. bergylta elovl5 and fads2

Primers for amplification of the first fragment of *elovl5* cDNA were designed on conserved regions of the following *elovl5* sequences: *Sparus aurata* (NCBI accession: AY660879), *Oreochromis niloticus* (NM_001279460), *Lates calcarifer* (GQ214180), *Clarias varegatus* (XM_015374479), *Dicentrarchus labrax* (FR717358), *Pagrus major* (HQ415605), *Siganus canaliculatus* (GU597350), *Siniperca chuatsi* (EU683736), and *Larimichthys crocea* (NM_001303374). The first fragment was amplified using LBElovlF2 and LBElovlR2 (Table 1) with GoTaq® Green Master Mix (Promega, USA). The PCR for the first fragment amplification consisted of 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min, and 72 °C for 5 min. The amplified fragment was purified on an agarose gel using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare UK Limited, UK) then sequenced (GATC Biotech, Germany). The obtained sequence was subsequently used for designing primers to perform 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) (Table 1). The RACE cDNA was synthesised using the FirstChoice™ RLM-RACE Kit (Thermo Fisher Scientific) following the manufacturer's instructions. All the PCR amplifications (first and nested) were performed with 95 °C for 2 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and 72 °C for