Aquaculture 416–417 (2013) 48–56

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Characterization of two Δ^5 fatty acyl desaturases in abalone (*Haliotis discus hannai* Ino)

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ARTICLE INFO

Article history:
Received 11 June 2013
Received in revised form 26 August 2013
Accepted 27 August 2013
Available online 3 September 2013

Keywords:
Fatty acyl desaturase
Long chain polyunsaturated fatty acid
Marine invertebrate
Fatty acid metabolism

ABSTRACT

Long chain polyunsaturated fatty acid (LC-PUFA) biosynthesis is limited in many marine species, normally due to lack of Δ^5 fatty acyl desaturase (Fad) activity. Among exceptions, abalone possesses considerable LC-PUFA conversion ability from C18 precursors. However, its characterization and relevant enzyme are not well characterized. Here we successfully cloned and characterized two Δ^5 Fads in abalone (*Haliotis discus hannai* Ino). Two Δ^5 Fad mRNA transcripts, *Hdh*fad1 (GQ 470626) and *Hdh*fad2 (GQ 466197), were found in abalone sharing 96.82% similarity for cDNA sequence and 96.58% similarity for their deduced amino acid sequences. *Hdh*fad1 cDNA is 1530 bp in length with an opening reading frame (ORF) coding for 438 amino acids. *Hdh*fad2 cDNA is 1525 bp in length with an ORF encoding for 439 amino acids. Both have characteristic features of front–end desaturase, including three histidine boxes, an N-terminal cytochrome b5 domain with heme-binding motif and transmembrane regions. Both *Hdh*fad1 and *Hdh*fad2 were expressed in tissues of abalone, especially in hepatopancreas and intestine. *Hdh*Fad1 possessed higher Δ^5 desaturase activity but expressed at a lower level than *Hdh*Fad2. Both isoforms preferred 20:4n-3 than 20:3n-6 as substrate. These results should provide valuable information on the molecular evolution of Fads and better understanding of LC-PUFA biosynthesis in abalone.

1. Introduction

Polyunsaturated fatty acids (PUFA), such as linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), cannot be endogenously biosynthesized by human and so must be obtained in the diet. Both LA and ALA could act as precursors for the synthesis of arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which are long chain-polyunsaturated fatty acid (LC-PUFA) and essential to human health (Kompauer et al., 2005; Kuriki et al., 2007; Ruxton et al., 2005). However, such LC-PUFA cannot be synthesized by human at sufficient physiological rate and thus must be obtained through dietary supply, especially from aquatic products, therefore termed as the most biologically active essential fatty acids (EFA) (Olsen, 2011; Tacon et al., 2009).

For human beings, abalone is one of LC-PUFA suppliers with great potential. There are over 15 abalone species farmed in countries such as Australia, China, South Africa, USA and Mexico (Sales and Janssen, 2004; Su et al., 2004). Abalone can acquire and accumulate LC-PUFA mainly in two ways: from seaweeds rich in LC-PUFA that constitute their natural feed, and from its inherent ability of LC-PUFA biosynthesis from fatty acid precursors that has been suggested in previous studies (Durazo-Beltrán et al., 2003; Mai et al., 1996; Mateos et al., 2011, 2012a,b; Xu et al., 2004). Under circumstances of large-scale abalone mariculture, it is not preferred to use seaweed as feed because of its imbalanced trophic structure. Formulated diets have been widely used in abalone mariculture because of their stable supply, easy storage and good performance (Bautista-Teruel et al., 2011). In such diets, the source of LC-PUFA comes from fish oil (FO). However, with global supply of FO dwindled, vegetable oils, rich in C18 fatty acids but low in LC-PUFA, have been widely used to replace FO in aquatic diets (Myers and Worm, 2003; Peron et al., 2010; Tacon and Metian, 2008). This makes it especially important to find practical ways to stimulate LC-PUFA biosynthesis in abalone to keep its nutritional value.

As shown in Fig. 1, LC-PUFA biosynthesis from C18 PUFA is mediated by fatty acyl desaturases (Fads) and elongases (Tocher, 2003). In this metabolic pathway, Δ^5 Fad converts 20:3n-6 and 20:4n-3 to 20:4n-6 (ARA) and 20:5n-3 (EPA) respectively (Nakamura and Nara, 2004). Lack of Δ^5 activity is believed to be responsible for the low LC-PUFA biosynthesis in many teleost fishes (Castro et al., 2012; Mourente and Tocher, 1993; Seiliez et al., 2003; Tocher and Ghioni, 1999; Tocher et al., 2006; Zheng et al., 2009). To date, Δ^6 Fads (González-Rovira et al., 2009; Monroig et al., 2010; Ren et al., 2012; Seiliez et al., 2003; Tocher et al., 2006; Zheng et al., 2005, 2009) and elongases (Agaba et al., 2005; Gregory et al., 2010; Hastings et al., 2005; Morais et al., 2009; Zheng

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http://dx.doi.org/10.1016/j.aquaculture.2013.08.030

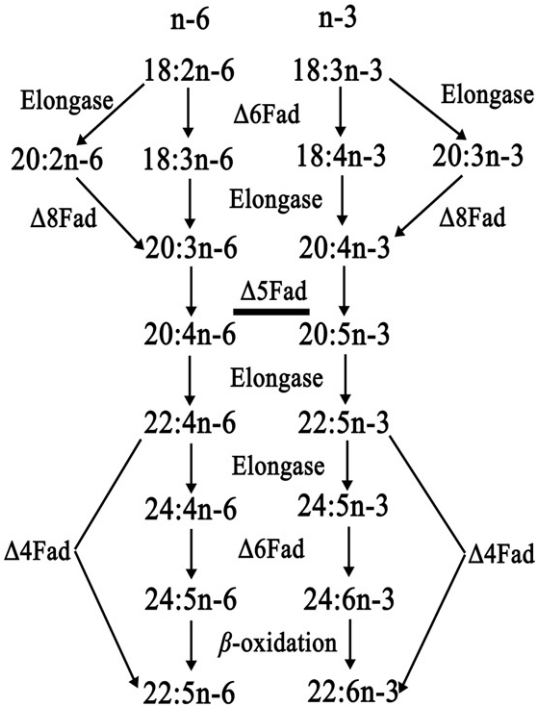

Fig. 1. Pathways for LC-PUFA biosynthesis (Nakamura and Nara, 2004; Tocher, 2003). ARA (20:4n-6) was biosynthesized from LA (18:2n-6) through n-6 pathway while EPA (20:5n-3) and DHA (22:6n-3) were produced from ALA (18:3n-3) through n-3 pathway. Δ^5 Fad is the direct enzyme for the biosynthesis of ARA and EPA.

et al., 2009) have been successfully cloned in many teleost fishes. However, attempts of Δ^5 Fad cloning in marine fishes have not been very successful (Seiliez et al., 2003; Tocher et al., 2006; Tu et al., 2012; Zheng et al., 2009). Previously, two bifunctional Fads ($\Delta^{5/6}$ and $\Delta^{5/4}$ Fads) were identified from white-spotted rabbitfish (*Siganus canaliculatus*) (Li et al., 2010). Recently, common octopus (*Octopus vulgaris*) Fad was reported to exhibit Δ^5 desaturation activity towards saturated fatty acid and PUFA (Monroig et al., 2011a). However, there is no identification about Δ^5 Fad or other Fads in any species of abalone.

This study was conducted to clone Δ^5 Fad in abalone, *Haliotis discus hannai* Ino. Furthermore, its enzymatic activity and tissue distribution were also investigated. These results will provide valuable information on the molecular evolution of Fads and better understanding of LC-PUFA biosynthesis in abalone.

2. Materials and methods

2.1. Materials (chemicals)

PrimeScript® 1st Strand cDNA Synthesis Kit, High Fidelity PrimeScript®RT-PCR Kit, High-fidelity Pyrobest® DNA Polymerase, PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time) and 2 × SYBR Green Real-time PCR Master Mix were obtained from TaKaRa (Shiga, Japan). SMART RACE cDNA Amplification Kit and Advantage 2 PCR Enzyme System were purchased from Clontech (Palo Alto, CA). pGEM-T Vector system was purchased from Promega (Madison, WI). *S.c.* EasyComp™ Transformation kit, Top10F' *E. coli* competent cells and restriction enzymes (Hind III and Xho I) were obtained from Invitrogen (Carlsbad, CA). Fatty acid methyl ester standards, raffinose, galactose, nitrogen base, tergitol type NP-40 and uracil dropout SD-U medium were gotten from Sigma-Aldrich (St. Louis, MO). Linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), eicosadienoic acid (20:2n-6), dihomo-γ-linolenic acid (20:3n-6), eicosatrienoic acid (20:3n-3), eicosatetraenoic acid (20:4n-3), docosapentaenoic acid (22:5n-3) and docosatetraenoic acid (22:4n-6) were purchased from Cayman Chemicals (Ann Arbor, Michigan).

2.2. Cloning of abalone Δ^5 Fad cDNA

Total RNA was extracted from freshly isolated hepatopancreas of abalone using TRIzol reagent followed by quality measurement on agarose gel electrophoresis and yield determination on NanoDrop® ND-1000 (Wilmington, DE). First strand cDNA was synthesized using PrimeScript® 1st Strand cDNA Synthesis Kit. To obtain the first fragment of Δ^5 Fad cDNA of abalone, degenerate primers were designed on histidine-conserved regions of Fads from different species using CODEHOP online software (http://blocks.fhcrc.org/blocks/codehop.html). PCR was conducted in Eppendorf Mastercycler Gradient (Eppendorf, Hamburg) using high-fidelity Pyrobest® DNA Polymerase. Cycling conditions were: 94°C for 2min followed by 35 cycles of 94°C for 30s, 60°C for 30s, 72°C for 60s, then with 5 min extension at 72°C. The PCR product (550bp) was separated by 2% agarose gel and the target band was obtained, gel purified, and then ligated into pGEM-T Vector. The PCR fragment was sequenced in Sangon Biotech (Shanghai) Co., Ltd. Specific primers were further designed for 3' and 5' rapid amplification of cDNA ends (RACE) to produce full-length cDNA. All primer sequences associated with open reading frame (ORF) cloning and RACE PCR were shown in Table 1.

First strand cDNA, used as template for 3' or 5' RACE PCR, was respectively produced by SMART RACE cDNA Amplification Kit. For 3' RACE, a positive fragment was obtained by two-round PCR. The program of first-round PCR was performed with an initial denaturing step at 94 °C for 2 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 2 min, and then extension at 72 °C for 5 min. Products from first-round PCR were used as template for nested-PCR in a 30-cycles reaction with the same thermal conditions mentioned above. As for 5' RACE, PCR was conducted following the instruction of Advantage 2 PCR Enzyme System. All PCR products were purified and sequenced as the way described above. Full-length cDNA sequences were deposited to the GenBank database.

2.3. Sequence and phylogenetic analysis

Deduced amino acid sequences of two newly cloned cDNAs were aligned with Δ^5 desaturases from different species including common octopus (*O. vulgaris*, AEK20864), white-spotted rabbitfish (*S. canaliculatus*, GU594278 and EF424276), zebrafish (*Dario rerio*, AAG25710), Atlantic salmon (*Salmon salar*, AAL82631), human (*Homo sapiens*, AAF29378), rat (*Rattus norvegicus*, AAG35068) and mouse (*Mus musculus*, BAB69894). Multiple sequence alignment was performed with MegAlign in Lasergene 7.0. Phylogenetic tree

Table 1
Primers used in Δ5 Fad full-length cloning, functional characterization and quantitative real-time PCR (qRT-PCR).

Aim	Primer	Primer sequence
ORF cloning	ORF-F	5′-CAACTGGTGGAACCACAGACAYTTYCARCA-3′[a]
	ORF-R	5′-GGAACAGGTGGTGCTCGATYTGRAARTT-3′[a]
3′ RACE	3RACE-outer	5′-TGCCAGTCCAGATTGCTAAAGAGAG-3′
	3RACE-inner	5′-ATTTCTTCCTCATCGGACCAC-3′
5′ RACE	5RACE	5′- CAAACAGTTCCTCCACCCTAACATCCG-3′
Functional characterization	HE-F	5′-CCC*AAGCTT*ACTATGGGCAAGGGAGGCCAAG-3′[b]
	HE-R	5′-CCG*CTCGAG*CTAGGGGCTGTGGAAGGCGTT-3′[b]
qRT-PCR	*Hdh*Fad1F	5′-CCATTTACTCCACCACATT-3′
	*Hdh*Fad1R	5′-AACAGTTCCTCCACCCTA-3′
	*Hdh*Fad2F	5′-CTGTTCAAGCCCAACG-3′
	*Hdh*Fad2R	5′-TTAAAGACGGAAATGTGAC-3′
	RPS9F	5′-GTCGGCTCGTGCGTAT-3′
	RPS9R	5′-GGATGTTCACCACCTGTTT-3′

[a] Y = C/T, R = A/G.
[b] Restriction sites (italic) for Hind III and Xho I respectively.