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Characterization, mRNA expression and regulation of Δ6 fatty acyl desaturase (FADS2) by dietary n − 3 long chain polyunsaturated fatty acid (LC-PUFA) levels in grouper larvae (*Epinephelus coioides*)

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ABSTRACT

N − 3 long chain polyunsaturated fatty acids (LC-PUFAs) are essential for marine fish to maintain normal growth and physiological functions, although the ability of LC-PUFA biosynthesis is limited. FADS2 was chosen to investigate its characterization, mRNA expression and regulation by dietary n − 3 LC-PUFA in the present study due to its being considered as the rate-limiting enzyme involved in LC-PUFA biosynthetic pathway. The FADS2 of grouper, when expressed in yeast, was shown to desaturate 18:3n − 3 and 18:2n − 6, indicating that it coded for a Δ6 desaturase enzyme. Lower desaturation of 20:3n − 3 and 20:2n − 6 indicated trace Δ8 activity. Following 4 weeks of feeding trial, the FADS2 mRNA expressions of grouper larvae that were fed diets with different LC-PUFA levels were measured and results showed that the expression of FADS2 increased significantly with dietary LC-PUFA from 0.52% to 0.94% and then decreased significantly ($P < 0.05$). The different growth performance and fatty acid composition of grouper among five treatment larvae also demonstrated that though the ability to synthesize LC-PUFA in grouper larvae was limited. These results showed that the FADS2 of grouper also displayed Δ8 desaturase ability when expressed in yeast, and dietary n − 3 LC-PUFA could regulate the biosynthesis of LC-PUFA through influencing the expression of FADS2.

1. Introduction

N − 3 long chain polyunsaturated fatty acids (LC-PUFAs) have been termed essential fatty acids (EFA) for marine fish, which are necessary for its normal development and functioning of nervous system and sensory organs (Benitez-Santana et al., 2007; Ishizaki et al., 2001). Meanwhile, n − 3 LC-PUFAs also play crucial role in structural components of cell membranes (Marsh, 2008), eicosanoids precursors (Bell et al., 2006; Tocher, 2003; Villalta et al., 2008), and regulator of gene expression (Calder, 2012; Deckelbaum et al., 2006). However, previous studies have shown that most marine fish, unlike freshwater species, need exogenous n − 3 LC-PUFAs to maintain normal growth, survival and physiological functions (Izquierdo et al., 1989; Kanazawa et al., 1979; Lee and Cho, 2009), which means that marine fish species have limited capacity to synthesize n − 3 LC-PUFAs de novo from their 18-carbon precursor fatty acids (Kanazawa et al., 1979; Tocher, 2003). N − 3 LC-PUFAs are also beneficial for human health, which could promote the development of neuronal tissues and protect against cardiovascular, immune and inflammatory condition (Calder, 2007; Salem et al., 2001). However, fish are the main source of LC-PUFAs for humans. Therefore, large amounts of research have drawn much attention on expression and activities of enzymes involved in LC-PUFA biosynthetic pathway.

The LC-PUFA biosynthetic pathway in fish was traditionally considered to proceed through consecutive desaturation and elongation reactions to convert C18 polyunsaturated fatty acids (PUFAs) linolenic acid (LNA, 18:3n − 3) and linoleic acid (LA, 18:2n − 6) to LC-PUFA, including docosahexenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) (Monroig et al., 2011). The Δ6 fatty acyl desaturase (FADS2) has been widely studied in fish since it was considered as the rate-limiting enzyme involved in LC-PUFA biosynthetic pathway (Hastings et al., 2004; Mohd-Yusof et al., 2010; Santigosa et al., 2011; Tocher et al., 2006; Zheng et al., 2009a). The enzyme is responsible for the step to convert 18:3n − 3 and 18:2n − 6 to 18:4n − 3 and 18:3n − 6, respectively. Meanwhile, it also involved in the "Sprecher shunt" pathway to produce DHA through EPA (Monroig et al., 2011; Sprecher, 2000). Monroig et al. (2011) found that FADS2 also displayed higher Δ8 activity than freshwater/diadromous species, which demonstrated a possible alternative "elongation–Δ8 desaturation–Δ5 desaturation" pathway. Monroig et al. (2011) also found that FADS2 activities of some marine fish are comparable with freshwater/diadromous species in yeast expression system, and this may indicate that the activity of FADS2 was enough for LC-PUFA biosynthesis of those marine fish species. However, to our knowledge no information is available on this assumption. Although the functional characterization and regulation of

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FADS2 have been widely studied on freshwater/seawater fish, no study has been conducted to determine the functional characterization and nutritional regulation of orange spotted grouper FADS2. One purpose of this study was to determine the activity of grouper Δ6 desaturase and whether the grouper putative FADS2 has the Δ8 desaturase ability. The nutritional regulation of FADS2 was also investigated to better understand the mechanism of LC-PUFA biosynthesis.

Orange spotted grouper, *Epinephelus coioides*, is a popular fish cultured in Southeast Asia and is a good candidate for intensive aquaculture of the region due to its fast growth, efficient feed conversion and high market value (Millamena, 2002). Lin et al. (2007) have reported that fish oil could be fully substituted by vegetable oils in fish meal-based diets for juvenile grouper without negatively affecting growth performance and feed utilization, which supposed that grouper may need a low level of n−3 LC-PUFAs or this LC-PUFA could be biosynthesized from ALA (18:3n−3) to some extent. However, as far as we know, no information was available on n−3 LC-PUFAs requirement and its biosynthetic ability of grouper. Compared with juvenile, fish larvae are much more sensitive to deficiency of dietary n−3 LC-PUFAs due to its important functions to maintain normal development and quick growth (Benitez-Santana et al., 2007; Bransden et al., 2005; Izquierdo, 1996). This difference makes fish larvae become a potential ideal model to study the ability of LC-PUFA biosynthesis, especially for some species with low n−3 LC-PUFAs requirement. Thus the present study was designed to investigate characterization, mRNA expression and regulation of FADS2 by dietary n−3 LC-PUFA levels in grouper larvae.

2. Materials and methods

2.1. Grouper

Grouper larvae were bought from a local fish rearing farm in Yandun, Hainan, China. The body mass of grouper used for cloning FADS2 was 10.0 g and the initial body weight of grouper larvae used for n−3 LC-PUFA level study was 70 ± 2 mg (29DAH).

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted from grouper liver using Trizol Reagent (Takara, Japan) followed by quality measurement on a 1.2% denaturing agarose gel and yield determination on NanoDrop® ND-1000 (Wilmington, DE). The RNA was treated with RNA-Free DNase (Takara, Japan) to remove DNA contaminant and reversely transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara, Japan) following the instructions provided by the manufacturer.

2.3. Functional characterization of grouper FADS2 by heterologous expression in Saccharomyces cerevisiae

PCR fragment corresponding to the open reading frame (ORF) of FADS2 was amplified from grouper liver cDNA using High Fidelity PrimeScript® RT-PCR Kit with primers containing restriction sites (underlined in Table 1). The DNA fragment containing the grouper FADS2 was digested with corresponding restriction endonucleases (Takara, Japan) and ligated into a similar restricted yeast episomal plasmid PYES2 (Invitrogen, UK). Then the purified plasmids containing FADS2 ORF were transformed to *S. cerevisiae* competent cells using S.c. EasyComp Transformation Kit (Invitrogen, UK). Transformation and selection of yeast with recombinant plasmids (pYES2–FADS2), and yeast culture were conducted as described in detail previously (Agaba et al., 2004; Li et al., 2013; Tocher, 2003). A single colony of transgenic yeast was grown in *S. cerevisiae* minimal medium$^{-uracil}$ supplemented with one of the following FA substrates: 18:3n−3 (0.5 mM), 18:2n−6 (0.5 mM), 20:3n−3 (0.75 mM), 20:2n−6 (0.75 mM), 20:4n−3 (0.75 mM), 20:3n−6 (0.75 mM), 22:5n−3 (1.0 mM) and 22:4n−6 (1.0 mM). The final concentrations of substrate FA were as described by Monroig et al. (2010). All the FA substrates (>98 ~ 99% pure) were purchased from Cayman Chemical Co. and chemicals used to prepare the *S. cerevisiae* minimal medium$^{-uracil}$ were from Sigma Chemical Co. Ltd. After 2 days' culture, yeast was harvested and washed twice by 5 mL ice-cold HBSS (Invitrogen, UK) and freeze dried for 24 h for further analyses. Yeast transformed with PYES2 containing no insert was grown under the same conditions as a control treatment.

2.4. Fatty acid analysis of cultured yeast

Fatty acid methyl ester (FAME) was prepared by the incubation of dried yeast samples with 1 N KOH–Methanol and then 2 N HCl–Methanol. After incubation, FAME was extracted by adding 1 mL hexane containing 0.01% BHT as antioxidant. FAMEs were separated and quantified using HP6890 gas chromatograph equipment with a fused silica capillary column (007-CW) and a flame ionization detector. The procedures for analysis of the fatty acid profiles were based on the method described by Metcalfe et al. (1966) with some modification (Ai et al., 2008). The column temperature was programmed to rise from 150 °C up to 200 °C at a rate of 15 °C min^{-1}, from 200 °C to 250 °C at a rate of 2 °C min^{-1}. Injector and detector temperature were 250 °C, respectively. Proportions of substrate FA converted to elongated FA product were calculated as [product area / (product area + substrate area)] * 100. The identities of fatty acid peaks and their double bond positions were performed by GCMS–QP2010Ultura (Shimadzu, Japan) as described previously (Agaba et al., 2004; Hastings et al., 2001).

2.5. n−3 LC-PUFA level study

Triplicate groups of grouper larvae (29DAH, 70 ± 2 mg) were fed to apparent satiation six times daily (07:00, 09:00, 11:00, 13:00, 15:00 and 17:00) for 4 weeks with five isoproteic (58% crude protein) and isolipidic (16% crude lipid) diets containing graded levels of n−3 LC-PUFA (0.52, 0.94, 1.57, 1.97 and 2.43%) (Tables 2 & 3). Prior to harvest, the fish were fasted for 24 h and weighted. Specific growth rate (SGR, percent per day) was calculated as 100 × [ln(final weight) − ln(initial weight)] / days. Visceral mass from five fish in each cage were pooled into 1.5 mL tube (RNase-Free, Axygen, USA), frozen in liquid nitrogen and then stored at −80 °C for later analysis of FADS2 expression. The remaining fish of each tank were pooled into 5 mL tube, frozen in liquid nitrogen and then stored at −80 °C for the assay of body composition and fatty acid composition.

Table 1
Details of primer pairs (restriction sites for HindIII and Xho I underlined) used for the cloning of grouper Δ6 ORF in pYES2, and qPCR primers.

Aim	Target gene	Primer	Primer sequence (5′–3′)	Reference[a]
ORF cloning	Δ6 FAD	H3FAF	CCCAAGCTTGGGAGGATGGGAGGTGGAGGCCA	GenBank: EU715405
		X1FAR	CCGCTCGAGCGGTCATTTATGGAGATATGCGT	
qPCR	Δ6 FAD	FAqF	CTCATCATTTGGGTCTGGG	GenBank: EU715405
		FAqR	GAAGATGTTGGGTTTAGCG	
	β-actin	ACqF	TACGAGCTGCCTGACGGACA	GenBank: AY510710
		ACqR	GGCTGTGATCTCCTTCTGCA	

[a] GenBank (http://www.ncbi.nlm.nih.gov/).