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Estimation of Tissue Distribution of mRNA Transcripts for Desaturase and Elongase Enzymes in *Channa striata* (Bloch, 1793) Fingerlings using PCR Technique

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ABSTRACT: Fish species are varied in their capacity to biosynthesize n-3 highlyunsaturated fatty acids (HUFA) such as eicosapentaenoic and docosahexaenoic acids (EPA & DHA) that are crucial to the health and well-being of all higher vertebrates. Experts report that HUFA metabolism involves enzyme-mediated fatty acyl desaturation (FAD) and elongation (FAE) processes. In previous studies, different workers cloned, characterized, identified and reported several genes for FAD and FAE enzymes in different fish species such as Atlantic salmon, gilthead seabream, rainbow trout and zebrafish, and also demonstrated the up- and down-regulation in the activity of these enzymes in response to fluctuations in dietary HUFA. In this paper, we report on the expression of genes (mRNA transcripts) for FAD and FAE enzymes in different tissues of *Channa striata* (Bloch, 1793) fingerling, to evaluate the tissues of the fish in which activity of both enzymes are high. To achieve this objective, we used conventional polymerase chain reaction (PCR) technique to isolate and quantify the absolute copy number for each gene transcripts from 8 different tissues of the fish (reared with a commercial feed). Our estimate show that the distribution of the 2 enzyme transcripts were significantly ($P < 0.05$) higher in the liver and brain of *C. striata* than detected in the 6 other tissues evaluated (muscle, ovary, testis, intestine, kidney and skin). Subsequently, we discuss here extensively, the implication of this observation with respect to the use of vegetable oils (VO) as substitute to fish oil (FO) in diets for freshwater fish species.

KEY WORDS: *Channa striata*, desaturase, fatty acid, gene, mRNA expression, PCR.

INTRODUCTION

Increasingly in recent times, the debate by researchers has become more intense on the suggestion that the supply of fish oil (FO), the common lipid source in fish feeds would not meet global demand in the near future (FAO, 2006). This is because recent research results reveal different vegetable oils (VO) as good and sustainable alternatives to FO in aquaculture. On the other hand however, concern also continues to trail the health benefits to humans who consume fish products reared with feeds formulated with VO (Bell et al., 2002; Robin et al., 2003; Torstensen et al., 2005; Tocher et al., 2006). Regardless of this development, research demonstrates that the only practical, sustainable alternative to FO is VO, which is a rich source of C18 PUFA although lacking in the n-3 HUFA abundant in FO (Sargent, 2002). The n-3 HUFA is however, crucial to the normal growth and development of all higher vertebrates (Simopoulos, 1989, 1991).

When VO is fed, tissue fatty acid (FA) composition of fish show characteristics of elevated C18 PUFA and reduced n-3 HUFA concentrations; leading to a much lower nutritional value of fish products to the human consumer (Bell et al., 2001). However, researchers discovered that some fish species possess the natural capacity to bio-convert dietary C18 PUFA precursors to HUFA, to increase their tissues' content of especially n-3 HUFA (Sargent et al., 2002; Kaushik, 2004). Consistent with this observation, Tocher (2003) specifically hypothesized that freshwater fish that are capable of this bio-conversion must express the activity of enzymes necessary for the process to occur. Important among these enzymes are fatty acyl desaturase and elongase (FAD and FAE).

Δ5 and Δ6 FAD and FAE are critical enzymes in the pathway for the biosynthesis of HUFA. In the last few years, significant progress has been made in characterizing FAD involved in HUFA synthesis (Tocher et al., 1996; Tocher et al., 1998). Authors have reported the isolation of a cDNA of zebrafish (*Danio rerio*, GenBank accession No AF309556), which showed high similarity to the mammalian Δ6 desaturase genes (Hastings et al., 2001). FAD cDNAs were also

isolated and cloned from rainbow trout (*Oncorhynchus mykiss*, GenBank accession No 56 AF301910) (Seiliez et al., 2001) and gilthead seabream (*Sparus aurata*, GenBank accession No AY055749) (Seiliez et al., 2003). Overall, the primary hypothesis is that the understanding of the molecular basis of HUFA biosynthesis and its regulation in fish would enable researchers to optimize the activity of the pathway. This would, ultimately, ensure the efficient and effective use of VO in aquaculture, while safeguarding the nutritional quality of reared fish for the human consumer.

To test the hypothesis of understanding the molecular basis for HUFA biosynthesis and regulation in fish therefore, we screened different tissues of *C. striata* fingerlings (maintained on a commercial diet) using molecular tools (PCR). This was to evaluate the potential distribution of the genes (mRNA transcripts) for the FAD and FAE enzymes in the species. The primers which we used in carrying out the screening were designed according to the published zebrafish FAD and FAE genes.

MATERIALS AND METHODS

Fish and collection of tissue samples

Snakehead fish, *C. striata* (Bloch, 1793) fingerlings (weight range of between 2–5 g) were maintained in a rectangular, 4000 L capacity fibreglass tank. Fish were fed a commercial, post-larval crumbled, catfish pellet (Post Larva 1 crumbles; Gold Coin Specialties Sdn Bhd, Johor, Malaysia) containing a minimum of 300 and 100 g/kg crude protein and lipid, respectively for 2 weeks prior to sampling. We fed the fish two times daily by hand to visual satiation except 24 hours prior to sampling when they were starved.

During sampling, we selected fish randomly with a hand-net, killed each immediately by a sharp blow to the head. Each was then immediately dissected using sterilized, standard dissecting kit, to remove 8 different tissues in triplicate (liver, brain, muscle, kidney, skin, intestine, ovary and testis). Tissue samples were separately stored in 2 mL of RNA later® and preserved at -80°C until used to extract total RNA within 3 days.

Total RNA extraction from tissues

To extract all the RNA from approximately 0.05 to 0.1 g of samples from each replicate pool of tissues (where appropriate), we used TRI Reagent® (Molecular Research Center, USA) and followed the manufacturer's specifications; all tissues were homogenized in 1 mL of TRI Reagent® (the skin and muscle were flash-frozen with liquid nitrogen before homogenizing). The homogenates were centrifuged at 12,000 g for 10 min at 4ºC to remove the insoluble material in the tissues. The supernatant was transferred to new, sterile 1.5 mL micro-centrifuge tube, into which 0.2 mL chloroform was added and subsequently shaken vigorously for 15 sec. The mixture was allowed to stand at room temperature for 15 min, after which it was centrifuged again at 12,000 g for 15 min at 4ºC. From 3 layers formed (upper clear aqueous phase, an intermediate phase and a lower red, phenol-chloroform phase), the upper aqueous phase was transferred to a new, sterile 1.5 mL micro-centrifuge tube and precipitated by the addition of 0.5 mL isopropanol. The sample was subsequently kept at -20ºC for 10–30 min (or overnight for some samples), followed by centrifugation at 12,000 g for 8 min at 4ºC. The supernatant was discarded, whereas RNA pellet formed was mixed with 75% ethanol to wash away any salts. This was centrifuged again at 7,500 g for 5 min at 4ºC. Ethanol was discarded carefully and the RNA pellet was air-dried at room temperature for 5–10 min. Finally, the RNA pellet was dissolved in appropriate quantity of RNase-free water and incubated on a water bath for 10 min at 55ºC to properly dissolve. The isolated total RNA was stored at -80ºC until used.

DNase treatment of total RNA

Subsequently, DNase treatment of all RNA extracted was carried out with RQ1 RNase-free DNase (Promega, USA), to eliminate all traces of DNA contamination in the RNA samples. The reaction was performed in a 10 µL mixture, containing 1 µg total RNA, 1 µL of RQ1 RNase-free DNase, 1 µL of 10X buffer (Promega, USA) and distilled water. The mixture was incubated at 37ºC for 30 min, followed by 65ºC for 10 min. Two micro liter of DNase-treated total RNA was reverse transcribed into first-stand cDNA at 42ºC for 1 h in a total volume of 20 µL containing 1 µL XM-MLV RT reaction buffer (Promega, USA), 25 ng of random primers, 0.5 mM of each dNTP, 24 U of recombinant RNasin® ribonuclease inhibitor and 200 U of M-MLV reverse transcriptase (Promega, USA). PCR was then performed to amplify the partial cDNA fragment of snakehead desaturase, elongase and ß-Actin (as housekeeping, reference gene), using primers from Primer3 online program (http://frodo.wi.mit.edu/cgi-bin /primer3/primer3.cgi/). The primers were designed according to published zebrafish desaturase (Genbank accession number: AF309556), zebrafish elongase (Genbank accession number: AF532782) and zebrafish β-actin (Genbank accession number: AF057040). The sequences of forward and reverse oligonucleotide primers we used and their amplicon sizes are shown in Table 1, which further verify the specificity of the primers used in the PCR analysis.

PCR for the expression of FAD, FAE and ß-Actin genes

Conventional polymerase chain reaction (PCR) was performed on the different tissues, to quantify the relative expression of mRNA transcripts of FAD and FAE enzymes in each tissue. In this step, 3 µL of first strand cDNA was added in a reaction mixture of 25 µL, containing Green Go Taq® Flexi buffer, 2.0 m*M* $MgCl_2$, 0.2 m*M* of each dNTP, 0.4 µ*M* of each primer and 1 U of Go Taq® DNA polymerase (Promega, USA). The PCR program used was an initial denaturation at 94ºC for 1 min; cycle amplification at 56ºC for 30 s (obtained from annealing temperature optimization carried out at between 50 and 62ºC for 30 s at each temperature, to verify the optimal annealing temperature) and 72ºC for 30 s with a final extension step at 72ºC for 10 min. The PCR products were separated by agarose gel electrophoresis containing 1% ethidium bromide, which were validated by running series of PCR reactions at different cycles to determine the number of cycles which generated maximal products. The cDNA bands for the FAD and FAE enzymes were visualized digitally by the use of CCDBIO 16SC imaging system (Hitachi Genetic Systems/MiraiBio, Alameda, CA). The visualized bands were quantified by densitometry (GENE TOOLS and GENE SNAP software, Hitachi Genetic Systems/MiraiBio), to calculate the ratio of the copy numbers of transcripts in each tissue relative to that for the reference (ß-actin) genes in the same tissue. The PCR assay was validated by running series of PCR reactions to determine the number of cycles which generate maximal PCR reaction, and were verified to be 32 cycles for FAD and ß-Actin and 33 cycles for the FAE genes accordingly.

Statistical Analysis

For tissue mRNA expression profiles, results are expressed as mean normalised values (± SD) corresponding to the ratio between the copy numbers of the FAD and FAE transcripts and the copy number of the reference gene, ß-Actin in the same tissues. One way analysis of variance (ANOVA) was carried out at a significance level of 0.05, followed by Duncan's Multiple Range post hoc Test, with SPSS statistical analysis software.

RESULTS

Expression of FAD and FAE genes in different tissues of *C. striata* fingerling

Fig. 1 shows the agarose gel chromatogram of the products of mRNA transcript bands for FAD and FAE enzymes in the 8 tissues of *C. striata* studied, whereas the PCR products of transcripts for the reference (ß-actin) genes in the same tissue are shown in Fig. 2. The normalised expression profile (mean ± SD, n=3), corresponding to the copy numbers of the FAD and FAE

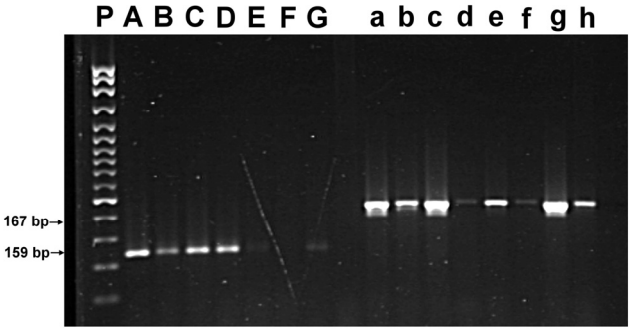

Fig. 1. Agarose gel chromatogram showing PCR products of mRNA transcript bands for FAD and FAE enzymes in 8 tissues of *Channa striata* fingerlings; Lane P, DNA molecular marker (1kb); Lanes A to H are bands for desaturase mRNA in liver (A), testis (B), brain (C), ovary (D), kidney (E), muscles (F), intestine (G) and skin (H); Lanes a–h, are bands for Elongase mRNA in liver (a), testis (b), brain (c), ovary (d), kidney (e), muscles (f), intestine (g) and skin (h).

transcripts in the liver, testis, brain, ovary, kidney, muscle, intestine and skin of *C. striata* fingerlings are as also shown in Fig. 3, accordingly.

The sequenced PCR product revealed that FAE (167 bp) and FAD (159 bp) derived from PCR analysis are 100% identical to the published zebrafish elongase (Genbank accession number: AF532782) and desaturase (Genbank accession number: AF309556), respectively, and further confirmed the specificity of the primers used in the PCR analysis.

From the results, expression of genes for FAD enzymes was similar in the liver, brain and ovary (numerically more widespread in the liver) and the normalized copy number was significantly ($P < 0.05$) higher than in other tissues tested. This was followed by testis, then the intestine, whereas expression of mRNA transcripts for FAD was recorded to be significantly the lowest ($P < 0.05$) in the skin. No expression of genes for FAD enzyme was detectable in both the kidney and muscle tissues, respectively.

Similar to the expression of genes for FAD enzyme, expression of transcripts for FAE enzyme was also significantly ($P < 0.05$) higher in the liver and brain, and the intestine as well [the expression did not significantly ($P > 0.05$) vary among the 3 tissues, but was numerically more widespread in the intestine]. This was followed by the kidney, testis and skin, where the expression of genes for FAE enzyme in these tissues was recorded at about half of that noted in the brain, liver and intestine, respectively. Expression for FAE enzyme was significantly ($P < 0.05$) the lowest in both ovary and muscle respectively, compared to the 6 others. Therefore, whereas expression for FAD enzyme was recorded in 6 tissues (liver, brain, ovary, testis, intestine and skin), the expression of transcripts for FAE enzyme was recorded in all the 8 tissues tested, although at varying levels for each of the enzymes.

Generally, expression of genes for FAE enzyme was recorded to be between 2–10 fold or more higher than that of genes for FAD enzyme in the tissues where both enzymes were detected, except in the ovary, where genes for FAD enzyme was recorded to be expressed at a higher level than that for the FAE.

DISCUSSION

Presently, no reasons are clearly attributed for the increase in HUFA biosynthesis in tissues, following limited intake of dietary HUFA (Tocher, 2003). This characteristic is even speculated to be species dependent (Jaya-Ram et al., 2008). Although we advance no evidence in this paper to correlate the obtained expression profile of mRNA transcripts with the activities of the enzymes, yet it is suggested that one of the most important mechanisms responsible for increased HUFA biosynthesis during limited dietary HUFA intake may be through the up-regulation in the expression of mRNAs for FAD and FAE enzymes (Bell et al., 2002). To support this hypothesis, there is emphasis by Tocher (2003) that freshwater fish and salmonids capable of producing DHA from 18:3n-3 must express the activity of the enzymes necessary for the biosynthetic pathway. This phenomenon was noted to occur in response to fluctuations in certain dietary nutrients (Sargent et al., 2002). Meanwhile, Breener (1981) showed that the activity of FAD enzymes is under nutritional regulation in mammals, and Tocher (2003) demonstrated that this occurs in fish as well, consistent with the observations which we made in different tissues of *C. striata* in this study.

Our observation in the current study, of significantly ($P < 0.05$) more widespread expression of genes for both FAD and FAE enzymes in the liver and brain of *C. Striata* concur with that in Zheng et al. (2009), where the authors also showed the highest FAD and FAE activities in both liver and brain of cobia (*Rachycentron canadum*). Furthermore, Tocher et al. (2006) also demonstrated the highest order of magnitude of desaturase activity in the brain of cod (*Gadus morhua,* L), with high levels also shown in the liver and kidney, followed by the intestine than in other tissues tested. Additionally, Tocher et al. (1996) earlier reported that 10-fold more DHA was produced from EPA than from 18:3n-3 in primary cultures of brain cells from turbot (*Scophthalmus maximus,* L). Similarly, expression of genes for desaturase enzyme was shown to be widespread in the liver and brain of a freshwater species rainbow trout, *Onchorynchus mykiss* (Seiliez et al., 2001), in Atlantic salmon, *Salmo salar* (Zheng et al., 2005) and Gilthead sea bream, *Sparus aurata* (Diez et al., 2007), but very low in kidney and white muscle of rainbow trout and in the intestine of sea bream.

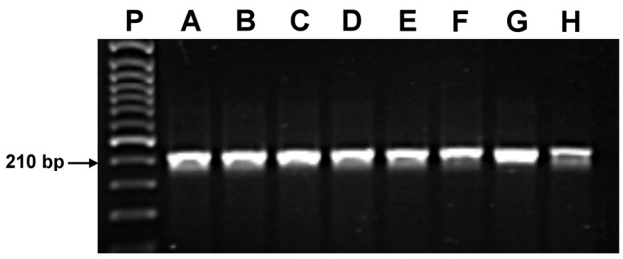

Fig. 2. Agarose gel chromatogram showing bands for PCR products for ß-Actin mRNA transcripts in 8 tissues of *Channa striata* fingerlings; Lane P, DNA molecular marker (1kb); Lanes A to H are bands for ß-Actin genes in liver (A), testis (B), brain (C), ovary (D), kidney (E), muscles (F), intestine (G) and skin (H)

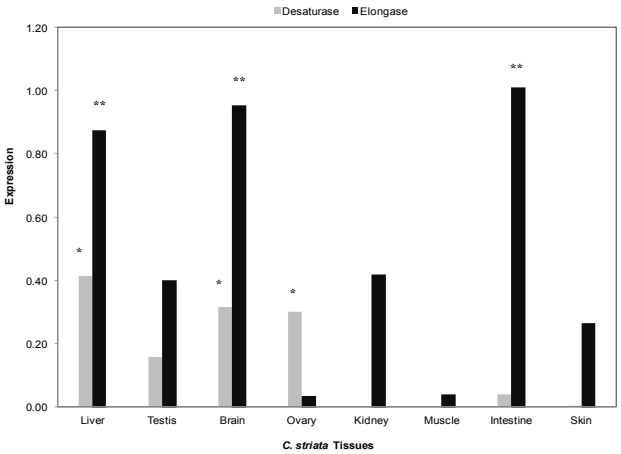

Fig. 3: Tissue expression profiles of mRNA transcripts for FAD and FAE enzymes in 8 different tissues of *C. striata* fingerlings. Expression levels were quantified for each transcript and normalized by absolute expression levels of the reference (ß-Actin) gene in the same tissue. Results are mean (n=3) ± SD; stars (* or **) on similar coloured bars imply significant differences ($P < 0.05$)

Higher gene expression for FAD and FAE enzymes observed in the brain and liver seem to suggest important roles in neural tissues, possibly to guarantee sufficient DHA despite fluctuations in the supply of dietary EPA and DHA, particularly during crucial periods of development, as suggested by Sargent et al. (2002). This speculation was confirmed by the work of Mourrente et al. (2003), who reported that during larval development, fish demonstrated considerable demand for DHA to develop neural tissues. Bell et al. (1995) also reported that larval Atlantic herring show impaired ability to capture prey at natural intensity of light due to a deficiency in dietary DHA. This supports the need for brain cells of fish to possess the capacity to produce DHA, in order to guarantee steady supply and avoid shortages from dietary sources.

Nearly all the cloned FAE genes which were functionally characterised from fish in the literature show similar sequence and functionality to that of the mammalian ELOVL5 (Hastings et al., 2005; Agaba et al., 2005). Theoretically therefore, only that Elovl5-like elongase may be required to produce DHA from 18:3n-3 in both fresh and marine water fish. This suggests that this one elongase is all required for the performance of both the elongations of EPA to produce DHA in freshwater and marine species, according to the hypothesis of Agaba et al. (2005).

Genes for enzymes in the HUFA biosynthetic pathway are also up-regulated in response to FO being substituted VO. In Atlantic salmon fed a linseed oil-based diet (containing high level of 18:3n-3) for 20 weeks, the expression of genes for FAD and FAE enzymes positively and negatively correlated with dietary 18:3n-3 and n-3 HUFA contents, respectively (Leaver et al., 2008). On the contrary, Seiliez et al. (2003) observed that expression of liver desaturase mRNA transcripts in freshwater rainbow trout was not significantly affected by dietary VO, including linseed oil, rapeseed oil, olive oil and a blend of these oils.

Taken together, all these observations suggest that salmonids in the seawater phase (and freshwater fish in general) are capable of up-regulating the genes of enzymes in the HUFA biosynthetic pathway, and their corresponding activity in response to decrease in dietary HUFA. This in effect, highlights that these genes play significant roles in regulating the bio-availability of HUFA in different tissues, and at different concentrations based on need of the organism. Researchers need to properly understand this mechanism in different species, for the continued and sustained development of aquaculture industry.

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利用聚合酶連鎖反應技術估測線鱧仔稚魚組織內去飽和酶與延長酶 mRNA 轉錄產物之分布

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摘要：不同的魚種其合成n-3高度不飽和脂肪酸之能力也不同，這些高度不飽和脂肪酸如EPA和DHA對於高等脊椎動物的健康和生長都扮演重要的角色。眾多專業報告都指出高度不飽和脂肪酸涉及酵素媒介脂肪酸去飽和作用和脂肪酸延長作用。在過往研究中，不同的研究團隊都針對各魚種之FAD和FAE酵素基因做過複製、描述與鑑定，如大西洋鮭、金頭鯛、虹鱒魚和斑馬魚，這些研究也證實了以上酵素的活性調控與高度不飽和脂肪酸之攝取有關。本篇研究呈現了FAD和FAE酵素在線鱧仔稚魚體內不同組織的基因表現情形，並鑑定出體內兩種酵素都呈現高度活性的組織。為達到此目的，本研究使用了傳統聚合酶連鎖反應技術來分離及量化自魚體中八個不同組織分離出之基因轉錄體（該魚種以商業飼料飼養）。研究結果指出線鱧仔稚魚的FAD、FAE的酵素轉錄體分別在肝組織及腦組織擁用有比其他六種組織（肌肉、卵巢、睪丸、小腸、腎臟和皮膚）顯著更高的量（$P < 0.05$）。本文也就此次觀察結果，討論在淡水魚飼料中使用植物油作為魚油替代物之可行性。

關鍵詞：線鱧、去飽和酶、脂肪酸、基因、mRNA表現、聚合酶連鎖反應。