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Barramundi (*Lates calcarifer*) desaturase with Δ6/Δ8 dual activities

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Abstract

Barramundi is a commercially farmed fish in Australia. To examine the potential for barramundi to metabolise dietary α-linolenic acid (ALA, 18:3 n-3), the existence of barramundi desaturase enzymes was examined. A putative fatty acid Δ6 desaturase was cloned from barramundi liver and expressed in yeast. Functional expression revealed Δ6 desaturase activity with both the 18 carbon (C18) and C24 n-3 fatty acids, ALA and 24:5 n-3 as well as the C18 n-6 fatty, linoleic acid (LA, 18:2 n-6). Metabolism of ALA was favoured over LA. The enzyme also had Δ8 desaturase activity which raises the potential for synthesis in barramundi of omega-3 (n-3) long chain polyunsaturated fatty acids (LCPUFA) from ALA via a pathway that bypasses the initial Δ6 desaturase step. Findings from this study not only provide molecular evidence for the fatty acid desaturation pathway in the barramundi and also highlight the importance of taking extracellular fatty acid levels into account when assessing enzyme activity expressed in *Saccharomyces cerevisiae*.

Keywords: ALA, barramundi, Δ6 desaturase, extracellular fatty acids, lipid metabolism, n-3 LCPUFA

Introduction

Omega-3 (n-3) long chain polyunsaturated fatty acid (LCPUFA) particularly eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are important for normal growth and brain development in humans (Innis 2003; McCann and Ames 2005). This appears to be true also for fish where n-3 LCPUFA are required especially in larval development and deficiency can impair vision, cause low survival, poor growth rate and abnormal behaviours (Bell et al. 1995; Rainuzzo et al. 1997). The n-3 LCPUFA are supplied to farmed fish in fish oil and fish meal which have long been major ingredients of diets for fish husbandry. However, being heavily reliant on a declining global marine fish stock presents a risk to the aquaculture industry (Domergue et al. 2005; Glencross et al. 2007). The substitution of fish oil with n-3 containing vegetable oils could be a viable option for a sustainable and economical source of fat for fish feed production. However, the predominant n-3 fatty acid in vegetable oils, α-linolenic acid (ALA, 18:3 n-3), has 18 carbons (C18) and 3 double bonds, whereas EPA and DHA are longer with C20 and C22 and more unsaturated with 5 and 6 double bonds, respectively. Therefore, the use of ALA in aquaculture requires the ability of farmed fish species to elongate and desaturate this fatty acid.

It is generally recognised that freshwater fish are capable of converting both n-3 and n-6 C18 PUFA to their more unsaturated and elongated LCPUFA. On the other hand, marine fish are not efficient at these conversions and it is believed that this difference comes mainly from a deficiency or impairment of the Δ5 desaturase and also C18 to C20 elongation activities in marine teleosts fish (Tocher et al. 1989). These limitations have been suggested to have important implications for both LCPUFA biosynthesis in aquatic animals and their prospective dietary requirements in aquaculture (Glencross 2009). Diadromous fish such as barramundi (*Lates calcarifer*), which live in both freshwater and marine/estuarine environments are of

particular interest in relation to LCPUFA biosynthesis because of the known differences in dietary PUFA requirements and enzyme capabilities of converting PUFA to LCPUFA between marine and freshwater species. Barramundi is an important aquaculture species in Australia and many other Asian countries (Glencross 2006). Studies have been undertaken to examine the nutritional requirements of farmed barramundi and most of these studies suggest that this species requires fish meal as the predominant protein source, consistent with the carnivorouos/piscivorous nature of the fish (Catacutan and Coloso 1995; Glencross 2006; Williams et al. 2003). Some previous studies have shown that it is possible to replace a portion of the marine-derived protein/fats in the diets of juvenile barramundi with non-marine sources without compromising growth, feed conversion ratio or body protein and fat contents (Raso and Anderson 2003; Williams et al. 2003). However, all of these previous feeding trials included at least some fish meal or fish oil in all the experimental diets, making it difficult to draw clear conclusions as to the ability of these fish to derive n-3 LCPUFA from C18 PUFA precursors. Therefore, this study aimed to determine whether barramundi have the capacity to derive n-3 LCPUFA from short chain precursors in their diet. Understanding the molecular basis of LCPUFA biosynthesis and regulation in barramundi will allow us to optimise the activity of the pathway to enable effective utilization of vegetable oil-based fish diets in aquaculture while maintaining the LCPUFA status of the farmed fish that human consumed. In this study we present evidence that there is a fatty acyl desaturase (FADS) gene encoding active Δ6 desaturase in barramundi hepatocytes. Furthermore, heterologous expression and functional characterization of the enzymes revealed that the putative Δ6 desaturase has dual Δ6/Δ8 desaturase activity.

Materials and Methods

Chemicals

Organic solvents used in this study were all analytical grades from Ajax Finechem Pty Ltd (Auckland, New Zealand) or Chem-Supply (SA, Australia). Fatty acid substrates ALA, linoleic acid (LA, 18:2 n-6), eicosatrienoic acid (ETA, 20:3 n-3), eicosadienoic acid (EDA, 20:2 n-6), homo-γ-linolenic acid (DGLA, 20:3 n-6) and docosapentaenoic acid (DPA, 22:5 n-3) (all > 98-99% purity) were purchased from Cayman Chemical (MI, USA). Fatty acid substrate 24:5 n-3 was purchased from Larodan Fine Chemicals (Malmö, Sweden). Other chemicals and reagents were purchased from Sigma-Aldrich (MO, USA) unless specified otherwise.

Experimental fish

All experimental procedures were performed in accordance with institutional guidelines for the use of animals and the Australian code of practice for the care and use of animal for scientific purpose. The protocol was approved by the Animal Ethics Committee, University of Adelaide (Ethic number S-28-08). Australian juvenile barramundi were obtained from a commercial supplier (W. B. A. Hatcheries, SA, Australia).

RNA isolation

Total RNA was isolated from 10 mg of fish liver using a Qiagen RNeasy kit (Qiagen, Victoria, Australia) following the protocol provided by the manufacturer with the tissue initially disrupted using a Tissue Lyser (Mixer MM 300, F. Kurt Retsch GmbH & Co. KG, Haan, Germany). The quality and concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, USA), and the RNA integrity was confirmed by agarose gel electrophoresis.

Cloning of putative barramundi desaturase cDNA and sequence analysis

All primers in this study are summarized in ~~Table 1~~the Supplementary Table. FADS genes in fish and other animal species in the GenBank were aligned and the partial genes of desaturase was obtained by one-step reverse transcription polymerase chain reaction (RT-PCR) (OneStep RT-PCR Kit, Qiagen) with primers (PSfads2-F and PSfads2-R) designed from the conserved regions of the same genes of other species. The gene specific primers were then designed based on the partial gene fragments of the putative desaturase gene. The rapid amplification of cDNA end (RACE) protocol was modified from methods of Frohman (Frohman 1994) to obtain 3' and 5'end of the full length of barramundi desaturase gene. The cDNA for 3'RACE was reverse transcribed from total RNA by a hybrid primer (QtT) and incubating with reverse transcriptase (Omniscript RT Kit, Qiagen) at 42°C for 1 hr to generate a 3'end partial putative FADS sequence. PCR amplification was then performed using a primer containing part of the 3'end sequence (Qo-R) and a Fads3'raceGSP1-F with HotStar HiFidelity Polymerase Kit (Qiagen). PCR amplification was begun with an initial hot start enzyme activation step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56-60°C for 30 sec and extension at 72°C for 1 min and a final extension at 72°C for 10 min.

The second round of PCR (nested PCR) was performed using Fads3'raceGSP2-F and Qi-R primers to generate a more specific 3'end of FADS for BigDye terminator sequencing (Applied Biosystems automated 3730 sequencer) at Molecular Pathology Sequencing, Institute of Medical and Veterinary Science, South Australia. A similar approach was used in 5'RACE. To generate 5'end partial cDNA clones, reverse transcription was carried out using total RNA with the Fads5'raceRT-R primer to synthesize first strand products, and a polyA tail was then appended to the cDNA template using terminal deoxynucleotidyltransferase (TdT) (Invitrogen, Victoria, Australia) and dATP (Promega, NSW, Australia).

PCR amplification was performed using the QtT-F to form the second strand of cDNA and the Qo-F primer with the Fads5'raceGSP1-R used for reverse transcription. A nested PCR was carried out by using Fads5'raceGSP2-R primer with Qi-F primer to increase specificity of barramundi desaturase gene 5'ends. Both 3' and 5'RACE products of the desaturase gene were aligned with the partial gene fragments to identify the overlapping regions. Based on the RACE results, specific forward and reverse primers were designed for identifying the full sequence of the barramundi FADS including partial untranslated regions and FadsEcoRI-F and FadsXhoI-R were used to amplify the PCR product containing the putative FADS open reading frame (ORF). The putative barramundi FADS ORF was then purified, restriction digested and inserted into EcoRI and XhoI digest sites of the pYES2 vector (Invitrogen, USA) to yield the plasmid construct pYES2/BarraFADS. The coding region was successfully cloned (GenBank ID: GU047383) and then sequenced by pYES2-F and pYES2-R primers to confirm the sequence orientation and accuracy. The HotStar HiFidelity Polymerase Kit (Qiagen) was used throughout the cloning processes to minimize potential PCR errors.

Yeast transformation

Saccharomyces cerevisiae (*S. cerevisiae*, INVSc1 yeast strain from Invitrogen, USA) was transformed with pYES2/BarraFADS using the S.c. Easy CompTM Transformation Kit (Invitrogen). Transformant yeast cells containing the pYES2/BarraFADS plasmid were grown at 30°C and transformants were selected on synthetic minimal defined medium agar plates lacking uracil (SC selective plate^{-U}; SC-U plate) and supplemented with 2% glucose as the only carbon source. The transformant from a single colony was verified by DNA sequencing

Heterologous expression and incubation of fatty acids

An INVSc1 transformant colony containing pYES2/BarraFADS was grown overnight at 27°C in SC selective medium (SC selective medium^{-U}; SC-U medium) containing 2% glucose. Expression of heterologous barramundi FADS was induced by transferring log-phase yeast cells (OD_{600nm}=0.4) into SC-U medium containing 2% galactose and 0.25% tergitol. Cultures were then supplemented with fatty acid substrates from among the following: ALA, LA, ETA, EDA, 24:5 n-3 and DPA. Yeast cells transformed with pYES2/BarraFADS plasmid without galactose induction were used as negative controls. After 48 hr incubation, yeast cells were harvested and then washed with ddH_2O and total lipids were extracted and analysed as described below. At least three independent replicates of the yeast cultures with fatty acid substrate supplementation were performed.

Lipid extraction, methylation and gas chromatographic analysis of fatty acid methyl esters (FAME)

Total yeast cells for each treatment were harvested, dried, weighed and then resuspended in 1.5 ml of 0.85% cold saline. One ml of medium from each cultured treatment was collected, weighed and 0.5 ml cold saline added. Free fatty acid 17:0 (0.4 mg/ml) (Nu-Chek Prep Inc, MN, USA) was added into each extraction tube as an internal standard. Total lipids were extracted from the cells by vigorous vortexing in chloroform-isopropanol (2:1, v/v) following a modification of the protocol of Bligh and Dyer (Bligh and Dyer 1959). The resulting chloroform phase was evaporated to dryness under nitrogen gas, and the lipids were transferred into a vial containing 1% sulphuric acid (H_2SO_4) in methanol at 70°C for 3 hr for transmethylation. All solvents used for extraction and separation contained 0.005% (w/v) antioxidant, butylated hydroxyanisol (BHA). After the samples were cooled, the resulting FAME were extracted with *n*-heptane and transferred into vials containing anhydrous sodium sulphate (Na_2SO_4). FAME were separated and quantified by GC (Hewlett-Packard 6890, CA, USA) equipped with a capillary

column (50 m x 0.32 mm id) coated with 0.25 µm film thickness 70% cyanopropyl polysilphenylene-siloxane (BPX-70, SGC Pty Ltd, Victoria, Australia) and a flame ionisation detector (FID). The injector temperature was set at 250°C and the FID temperature at 300°C. The oven temperature at injection was initially set at 140°C and was programmed to increase to 220°C at a rate of 5°C per minute. Helium gas was utilized as a carrier at a flow rate of 35 cm per second in the column and the inlet split ratio was set at 20:1. The identification and quantification of FAMEs were achieved by comparing the retention times and peak area% values of unknown samples to those of commercial lipid standards (Nu-Chek Prep Inc, MN, USA) using the Hewlett-Packard Chemstation data system. The actual weight of fatty acid traces was computed by comparing the area of the peak associated with the internal standard (17:0) with the area of the peak of the individual fatty acid in samples. FAME standards stearidonic acid (SDA, 18:4 n-3) and 20:4 n-3 were purchased from Cayman Chemicals. FAME standards 24:5 n-3 and 24:6 n-3 were purchased from Larodan Fine Chemicals.

Statistical analysis

All data are expressed as group mean ± SEM. A one-way analysis of variance (ANOVA) followed by Tukey-HSD test was used if *P* value less than 0.05 and follow Gaussian distributions (calculated by the Kolmogorov and Smirnov test). Kruskal-Wallis post test with Dunn's multiple comparison tests was applied for non-parametric analyses if data does not pass the normality test. An unpaired t-test was used to examine difference between two groups. A probability level of 0.05 ($P < 0.05$) was used in all tests. Analyses were carried out with GraphPad Instat version 3.10 (GraphPad Software, CA, USA) for Windows.

Results

Sequence analysis

Sequence data from the cDNA clone indicated that the putative barramundi FADS included an ORF of 1338 bp nucleotides which coded for a protein of 445 amino acids. Parallel alignment of the putative barramundi desaturase enzyme sequence with Δ5 and Δ6 desaturase peptide sequences from human, baboon, rat, zebra fish and Atlantic salmon indicated that the barramundi putative desaturase gene contained elements typical of a membrane-bound desaturase. These include a HPGG heme binding motif in an N-terminal cytochrome b_5-like domain, three histidine-rich sequences (His boxes) comprised of a group of eight conserved histidines: HPGG, HDXGH, HFQHH and QIEHH and four transmembrane regions: N_{130}-RPLFFCLHLGHIVLLEALAWLMI-C_{152}, N_{154}-LWGTNWILTSLCAVMLATAQSQ-C_{175}, N_{267}-FFLVGPPLLIPVFFHIQIMHTMI-C_{289} and N_{309}-SCYIPLYGLFGSLALISFVRFLE-C_{331} (Fig. 1). The barramundi putative desaturase has higher sequence identity to human (*Homo sapiens*) (65%) and rat (*Rattus norvegicus*) (64%) Δ6 desaturase (FADS2) than human (51%) and rat (57%) Δ5 desaturase (FADS1). The putative barramundi desaturase also showed 65% sequence identity to a baboon (*Papio anubis*) Δ6 desaturase. When comparing the peptide sequence homology with Atlantic salmon (*Salmon salar*), the deduced barramundi putative desaturase was found to share 77% identity with FADS1 and 76% with FADS2 (Table ~~2~~1). In addition, alignment of the barramundi putative desaturase peptide sequence in this study with another recently reported barramundi putative Δ6 desaturase (GQ214179) showed 98% homology and identity in the peptide sequence, but differed in 6 amino acids (Pro 95 Leu, Arg 149 Trp, Pro 317 Leu, Thr 346 Met, Ser 366 Asn and Arg 377 Ser) throughout the coding region (Fig. 1). The barramundi putative desaturase gene coding region that we isolated from juvenile fish using 3'/5' RACE by degenerate and gene specific primers had overlapping regions of a given cDNA fragment, the deduced putative full length ORF sequence was confirmed by the cloning and sequencing, and we are therefore confident for the accuracy of the sequence. The deduced peptide sequence of FADS

in this study has a molecular weight of 51.8 kilodaltons which was predicted by Science Gateway Protein Molecular Weight Calculator (http://www.sciencegateway.org/tools/proteinmw.htm).

The recombinant barramundi desaturase enzyme has both Δ6 and Δ8 activity

The barramundi putative desaturase gene was functionally characterized by expression in transformed yeast *S. cerevisiae* containing plasmid pYES2/BarraFADS and induced by SC-U medium containing 2% galactose. In cells there was a concentration dependent and time-dependent increase up to 48 hr in yeast lipid SDA, indicating Δ6 desaturase activity (Fig. 2 a). There was no detectable SDA secreted into the medium (Fig. 2 b). Δ6 Desaturase activity was also observed with 24:5 n-3 and with the n-6 substrate, LA (Table ~~5~~3). Some fatty acid products of the desaturase activity were found in the medium as well as in the yeast and therefore, these were taken into account when calculating the percentage conversion of substrate. The percentage conversion of LA was about half that of the equivalent n-3 substrate, ALA (Tables ~~3~~ 2 and ~~5~~3). We investigated other potential desaturase activities by adding the appropriate fatty acids. Δ8 Desaturase activity was observed with both n-3 (ETA) and n-6 (EDA) substrates (Tables ~~3~~ 2 and ~~5~~3). Once again, the recombinant enzyme was more active with n-3 than n-6 substrate. No Δ4 or Δ5 desaturase activity was observed with the addition of the potential substrates (Table ~~5~~3).

Induction of the barramundi FADS ORF in yeast without exogenous fatty acids did not alter the concentration of any of the major endogenous fatty acids, indicating no desaturase activity with saturates and monounsaturates (Fig. 4).

Desaturation products in the culture medium

For the C18 fatty acids, only trace amounts of LA and ALA substrates were present in the medium after 48 hr of culture (Table 4) and only trace of ALA could be detected at 6 hr (Fig. 2 b). Neither of the desaturated products γ-linoleic acid (GLA, 18:3 n-6) and SDA, were detected in the medium. In contrast, significant amounts of EDA, ETA and 24:5 n-3 substrates were still present in the culture medium after 48 hr (Table 4). In addition, the desaturated products 20:4 n-3 and 24:6 n-3 from both n-3 substrates (ETA and 24:5 n-3, respectively) were detected in the culture medium (Table 4). Therefore the desaturation product concentrations present in the culture medium, as well as those present in the cells, were taken into account for calculating the apparent conversion rate (Table ~~5~~3). When this was done, the conversion of ALA to SDA, LA to GLA and EDA to DGLA remained unchanged but the conversion of both ETA to 20:4 n-3 and 24:5 n-3 to 24:6 n-3 increased 6.9-fold.

Dose-response of ALA on the effects of the desaturase activity

Although accumulation of ALA in cells was concentration dependent (Fig. 3 a), the SDA amounts detected still only represented a small percentage (2%-6%) of the total ALA added to the culture (Fig. 3 b). The accumulation of SDA was not strictly related to cell ALA levels.

Discussion

Conversion of the plant derived ALA to produce SDA, generally recognised as an intermediate in n-3 LCPUFA synthesis, requires Δ6 desaturase activity. This study has isolated a Δ6 desaturase in barramundi that is able to utilise C18 n-3, n-6 and C24 n-3 as substrates. The ability to desaturate 24:5 n-3 is important because this fatty acid is an intermediate in DHA synthesis via an indirect or shunt pathway, at least in rat and human tissue (Sprecher et al. 1999). An apparent direct pathway to DHA synthesis from EPA would involve elongation to DPA and then Δ4

desaturation to DHA. A recent report indicated that a marine species *Siganus canaliculatus* FADS2 and FADS1 displays bifunctional Δ4/Δ5 and Δ6/Δ5 desaturase activities, respectively (Li et al. 2010). Another Δ4 desaturase which can convert DPA directly to DHA has been identified in *Thraustochytrium sp*., a common marine fungus which produces DHA (Lewis et al. 1999; Qiu et al. 2001). However, Δ4 desaturase activity has not been found in rats and humans and the barramundi Δ6/Δ8 desaturase described in this study showed no Δ4 activity. In rats and humans there is evidence that DPA is elongated to 24:5 n-3 which is converted by Δ6 desaturation to 24:6 n-3 and then chain-shortened by β-oxidation to DHA (Sprecher et al. 1999). There is evidence that this may occur also in at least one fish species, gilthead sea bream, where radio-labelled 24:5 n-3 and 24:6 n-3 were found after injection of radio-labelled ALA or EPA (Mourente and Tocher 1994). The fact that the Δ6 desaturase isolated in this study is capable of desaturating 24:5 n-3, suggests this pathway identified in mammalian tissue and suggested in gilthead sea bream, could also be active in barramundi.

The dual n-3 C18/C24 activity and Δ6/Δ8 desaturase activity of the putative desaturase enzyme in barramundi raises the possibility of competition between ALA and 24:5 n-3, as well as Δ6/Δ8 substrates. Increasing ALA concentrations in HepG2 cells resulted in increasing and then decreasing DHA production, suggesting competition between n-3 substrates for Δ6 desaturase at the higher concentrations of ALA (Portolesi et al. 2007).

Δ6 Desaturase activity has been identified in other fish species, and an enzyme in zebra fish exhibited both Δ5 and Δ6 desaturase activity (Hastings et al. 2001). However, the recombinant barramundi desaturase in this study had no detectable Δ5 desaturase activity. Δ6 desaturases from carp, turbot (Zheng et al. 2004), rainbow trout (Seiliez et al. 2001) and gilthead sea bream (Seiliez et al. 2003) also had little

or no Δ5 desaturase activity (Zheng et al. 2004). Δ5 Desaturase is necessary for n-3 LCPUFA synthesis and in mammals, Δ6 and Δ5 desaturase activities reside in separate genes; namely, FADS2 and FADS1, respectively. To date, the only unifunctional Δ5 desaturase in fish has been characterized in Altlantic salmon (Hastings et al. 2004). We used degenerate primer sets designed from consensus regions of known Δ5 desaturase cDNA in animals and Salmonid to probe for barramundi Δ5 desaturase cDNA but without success (data not shown). The partial nucleotide sequence (~850 bp) that we obtained from the barramundi cDNA pool using Δ5 desaturase primers was 100% matched with the partial sequence of the desaturase that we have isolated in this report. There is higher than 90% homology in peptide sequences between Atlantic salmon Δ5 and Δ6 desaturases. If this is the case also with barramundi enzymes, the primers designed for Δ5 desaturase may have preferentially recognised Δ6 desaturase cDNA if Δ5 desaturase mRNA levels were very low relative to those of Δ6 desaturase. Therefore, we are unsure whether there is little or no expression of Δ5 desaturase mRNA or no presence of the gene at all, in barramundi. Nevertheless, it appeared that if this gene is present, it is at much lower levels than Δ6 desaturases.

The barramundi Δ6/Δ8 desaturase was able to desaturate both n-6 and n-3 substrates. The enzyme exhibited approximately twice the desaturation activity with C18 n-3, compared with C18 n-6 substrates, suggesting that n-3 LCPUFA synthesis could proceed efficiently even in the presence of an equivalent concentration of homologous n-6 substrates. Glencross and Rutherford (Glencross and Rutherford 2011) reported that the retention efficacy of barramundi tissue ALA decreased with decreasing inclusion levels of dietary DHA, EPA and arachidonic acid (AA, 20:4 n-6), suggesting desaturation and elongation of short chain precursors is occurring. However, while the addition of AA to the diet increased the tissue EPA retention level, the inclusion of equivalent amounts of EPA in the diet showed no effect on

tissue AA retention level. This indicates the potential negative effect of n-6 LCPUFA on EPA utilisation by other enzymes in the LCPUFA pathway of barramundi. In addition, in the current study, the results of the supplementation of yeast cells with increasing concentrations of ALA indicates that the degree of incorporation of the substrate ALA and desaturation product SDA was not equivalent (Fig. 3), and this may be a result of saturation of the enzyme with substrate. Thus, different apparent conversion rates will result at different substrate concentrations. The curvilinear relationship of the substrate ALA level and the conversion of SDA from ALA is similar to the DHA levels reported in rats (Tu et al. 2010) and piglets (Blank et al. 2002) with increasing dietary ALA levels.

Although the metabolism of ALA by Δ6 desaturase is considered the principal first reaction in its conversion to EPA via SDA and then 20:4 n-3, there is some evidence of an alternate or bypass pathway involving Δ8 desaturation. If ALA is elongated to ETA, this could be a substrate for 20:4 n-3 synthesis via Δ8 desaturation. This reaction has been shown to exist in rat and human testicular tissue, but not rat liver (Albert et al. 1979; Albert and Coniglio 1977). Baboon hepatic Δ6 desaturase expressed in yeast also has Δ8 desaturase activity, although it was 23-fold and 7-fold lower than Δ6 desaturase activity for n-3 and n-6 substrates, respectively (Park et al. 2009). Apart from the present barramundi enzyme, which we observed to have Δ8 desaturase activity with n-3 and n-6 substrates, none of the reported fish Δ6 desaturases have been examined for Δ8 desaturase activity (Hastings et al. 2001; Hastings et al. 2004; Mohd-Yusof et al. 2010; Tocher et al. 2006; Zheng et al. 2004; Zheng et al. 2005) until recently, Monroig and others (Monroig et al. 2011) re-examined several Δ6 desaturases in freshwater, diadromous and marine fish species for Δ8 desaturase activity and they suggested that the Δ8 is also a characteristic of various fish. In this present study, the Δ8 desaturase activity of the barramundi Δ6/Δ8 dual functional enzyme was 3.9-fold

higher than the Δ6 desaturase activity with ALA but 2.8-fold lower than LA substrates. The fold difference was derived from the conversion (%) which has been taken the extracellular product levels into account. Thus, it had a higher preference for Δ8 than Δ6 activity when encountering ETA substrate (Table ~~5~~3).

Another recombinant barramundi FADS gene has recently been isolated with a conversion rate of 32% from ALA to SDA and 28% towards LA to GLA (Mohd-Yusof et al. 2010). This desaturase activity is substantially higher than the activity that we have reported here. However, direct comparisons with the other barramundi enzyme are difficult when fatty acid substrate concentrations were not stated, only conversion values and no mass data were reported, and the incubations were for 3 days (Mohd-Yusof et al. 2010; Zheng et al. 2009). If the differences in enzyme activities are real, they may be due to the 6 amino acid difference among the full ORF between the two genes. The results suggest there will be polymorphism in FADS gene in the two barramundi population, due to either the presence of the FADS2 isoforms or regional variation (Ward et al. 2008) of barramundi species.

One of the major findings of this study has been to highlight the limitation of the yeast cell culture system for quantitatively assessing enzyme activity. We identified two key obstacles, namely (1) the rapid loss of some substrates presumably by ß-oxidation (2) the significant release of some desaturation products to the culture medium. For example, C20 and C24 fatty acids were detected in the media at the end of incubation period but C18 fatty acids had all been utilised by 6 hr indicates a differential intake capacity of yeast which is dependent on fatty acid chain length and saturation status. As a result, we concluded that any desaturation products which were present in the medium needed be taken into account in any calculation of enzyme activity. When we added together the total amount of desaturation products in both the cells and medium, we observed the conversion rates of ETA to

20:4 n-3 and 24:5 n-3 to 24:6 n-3 were changed accordingly (Table ~~5~~3). Moreover, care must be taken when comparing enzyme activities of enzymes such as desaturases in other species because C18 fatty acid like ALA and LA may appear to be more favoured by yeast expression system than C20 and C24 substrates (Table 4). The less efficient incorporation of C20, C22 and C24 fatty acid into yeast cells than C18 has also been addressed by van Roermund and others (Li et al. 2010; van Roermund et al. 2003).

In conclusion, heterologous expression of barramundi FADS2 gene in yeast demonstrates the coding enzyme shows Δ6/Δ8 dual functionality and exhibits a preference for n-3 over n-6 fatty acid substrates. The availability of substrates for the Δ8 desaturase activity would depend on the presence of the C20 fatty acid substrates in the food chain or the occurrence of an elongase that could convert ALA to ETA or LA to EDA. From the same barramundi, we have also cloned and characterised an elongase (GenBank ID: GU047382) that is capable of elongating ALA and LA to ETA and EDA respectively. According to this result, we postulate that the LCPUFA production from C18 precursors in barramundi can be processed through either Δ6 or Δ8 desaturation pathways (Fig. 5). However, it is still unclear whether barramundi has a Δ5 desaturase for desaturation of 20:4 n-3 and DGLA. If it is expressed, it is likely to be at much lower levels than the Δ6 desaturase. Moreover, in the whole animal where all substrates can be present at the same time, there is the potential for n-3/n-3 and n-3/n-6 substrate competition which would affect pathway flux.

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Figure legends

Fig 1. Alignment of the predicted peptide sequences of the putative barramundi desaturase (GU047383, this study) with desaturases in human, baboon, rat, zebra fish, and salmon. Identical amino acids are in black, the heme binding motif in cytochrome b_5-like domain is framed with a square; three histidine-rich domains are underlined. Four putative transmembrane regions (dashed line) are predicted by SOSUI software http://bp.nuap.nagoya-u.ac.jp/sosui/. *Putative barramundi Δ6 desaturase from Mohd-Yusof *et. al.* (GQ214179) (Mohd-Yusof et al. 2010).

Fig 2. Time course for SDA production after supplementation of ALA (250 μM) in transformed yeast cells (total mass of cells) (a) and medium (b) after expressing barramundi FADS ORF. All values are means ± SEM, n=3 (independent experiment). Total amount of fatty acids in the yeast is calculated as μg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

Fig 3. Dose-response of ALA on effects of barramundi desaturase activity. Effects of increasing ALA at concentrations up to 500 μM on synthesis of SDA (a) and on the conversion (%) of ALA to SDA (b). Desaturation products were quantitatively computed as μg of fatty acids of total cells per culture. Conversion (%) was calculated by conversion (%) = [product / (substrate + product) × 100]. Mean ± SEM, n=3 (independent experiment). Means superscripted with different letters indicate statistical difference ($P \leq 0.05$), according to Kruskal-Wallis post test with Dunn's multiple comparison test.

Fig 4. Fatty acid profiles of total lipid in uninduced (solid bar) and induced (open bar) yeast cells containing barramundi FADS ORF. Fatty acid contents were

quantitatively calculated based on peak area of an internal standard (C17:0). The data was represented as mean ± SE, n=6 (independent experiment).

Fig 5. A postulated LCPUFA biosynthetic pathway for barramundi.

Table ~~2~~ 1 Identity comparison of FADS peptide sequences of barramundi and other species

	Barramundi FADS2[a] (GQ214179)	*Human* FADS1 (NP_037534)	*Human* FADS2 (AAG23121)	*Baboon* FADS2 (ACI46980)	*Rat* FADS1 (BAB69054)	*Rat* FADS2 (BAA75496)	*Zebra fish* FADS2 (AAH49438)	*Atlantic salmon* FADS1 (AAL82631)	*Atlantic salmon* FADS2 (AAR21624)
Barramundi FADS2 (this study) (GU047383)	98%[b]	51%	65%	65%	57%	64%	69%	77%	76%

[a]The peptide sequences were derived from coding sequences deposited at the NCBI GenBank database.

[b]Sequences were aligned and the identity was computed using the Genedoc (National Resource for Biomedical Supercomputing, NRBSC).

Table ~~3~~ 2 Fatty acid composition of yeast cells expressing barramundi FADS ORF

µg/total cell	18:2 n-6 (LA) (500 µM)		18:3 n-3 (ALA) (500 µM)		20:2 n-6 (EDA) (500 µM)		20:3 n-3 (ETA) (500 µM)		24:5 n-3 (100 µM)	
Fatty acid	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced
16:0	27.1±4.7[a]	25.9±0.7	16.6±2.4	29.6±1.7	32.1±7.1	29.4±2.5	25.4±1.9	25.2±1.0	15.1±0.4	31.2±12.0
18:0	12.1±1.9	11.7±0.3	8.6±1.1	14.05±0.5	13.8±2.5	12.9±0.9	12.5±1.5	11.4±0.3	8.6±0.4	15.6±4.9
16:1n-7	32.6±7.7	25.8±0.2	17.0±3.4	27.2±1.5	53.8±16.0	39.0±4.9	32.4±0.1	31.2±2.9	25.4±0.6	54.2±24.7
18:1n-9	16.8±4.8	14.4±0.3	8.4±2.1	19.9±1.3	31.0±8.2	22.6±2.5	20.1±0.3	19.6±1.4	12.9±0.5	32.8±15.7
18:2n-6	24.2±5.2	19.2±0.1	-	-	-	-	-	-	-	-
18:3n-3	-	-	11.3±2.6	21.3±1.1	-	-	-	-	-	-
18:3n-6	-	0.2±0.0	-	-	-	-	-	-	-	-
18:4n-3	-	-	-	0.5±0.1	-	-	-	-	-	-
20:2n-6	-	-	-	-	29.3±6.3	31.1±1.7	-	-	-	-
20:3n-6	-	-	-	-	-	0.1±0.0	-	-	-	-
20:3n-3	-	-	-	-	-	-	12.4±0.5	19.2±1.6	-	-
20:4n-3	-	-	-	-	-	-	-	0.3±0.0	-	-
22:1n-9	15.6±0.6	15.1±1.2	13.5±0.6	15.0±1.1	11.7±3.0	9.2±2.2	7.7±0.7	13.6±2.3	15.6±0.8	16.1±1.4
24:5n-3	-	-	-	-	-	-	-	-	7.3±0.3	16.7±0.6
24:6n-3	-	-	-	-	-	-	-	-	-	0.2±0.0

[a]Desaturation products were quantitatively computed as µg of fatty acids of total cells (total mass of cells) per culture (10 ml). Mean ± SEM, n ≥ 3 (independent experiment)

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Table 3 Substrate specificity and enzyme activity

Fatty acid substrate[a]	Desaturation product	Conversion (%)		Enzyme activity
		Cell only[b]	Cell + medium[c]	
n-3 PUFA				
18:3 n-3 (ALA)	18:4 n-3 (SDA)	2.3±0.2	2.3±0.2	Δ6 desaturase
20:3 n-3 (ETA)	20:4 n-3	1.3±0.1	9.0±0.0	Δ8 desaturase
22:5 n-3 (DPA)	22:6 n-3 (DHA)	N.D.[d]	N.D.	Δ4 desaturase
24:5 n-3	24:6 n-3	1.2±0.1	8.3±0.6	Δ6 desaturase
n-6 PUFA				
18:2 n-6 (LA)	18:3 n-6 (GLA)	1.1±0.1	1.1±0.1	Δ6 desaturase
20:3 n-6 (DGLA)	20:4 n-6 (AA)	N.D.	N.D.	Δ5 desaturase
20:2 n-6 (EDA)	20:3 n-6 (DGLA)	0.4±0.1	0.4±0.1	Δ8 desaturase

[a]Fatty acids ALA, ETA, LA, DGLA and EDA were supplemented 500 μM, DPA was supplemented 250 μM and 24:5 n-3 was supplemented 100 μM into yeast culture medium as a final concentration.

[b]Fatty acid in yeast cells were used for calculating the conversion (%). Data are means ± SEM of $n \geq 3$. Conversion (%) = [cellular product / (cellular substrate + cellular product) × 100]; all fatty acids were calculated based on fatty acid amount listed on Table ~~3~~2.

[c]Desaturation products in medium were taken into account for calculating the conversion (%). Data are means ± SEM of $n \geq 3$. Conversion (%) = [cellular and extracellular product / (cellular substrate + cellular and extracellular product) × 100]; all fatty acids were calculated based on fatty acid amount listed on Tables ~~3~~ 2 and 4.

[d]N.D. = not detected.

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Table 4 Fatty acid composition of remaining cell-free medium from yeast culture expressing barramundi FADS ORF

µg/total medium	18:2 n-6 (LA) (500 µM)		18:3 n-3 (ALA) (500 µM)		20:2 n-6 (EDA) (500 µM)		20:3 n-3 (ETA) (500 µM)		24:5 n-3 (100 µM)	
Fatty acid	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced
16:0	107.2±2.2[a]	174.75±3.5	160.4±6.0	147.3±5.5	154.65±1.3	154.95±9.1	143.1±5.3	146.3±5.9	104.3±3.3	112.1±0.2
18:0	96.9±1.9	144±2.5	132.73±4.3	122.63±3.9	125.6±0.8	125.1±5.9	120.2±3.6	120±3.8	93.6±2.3	99.5±0.3
18:1n-9	4.5±0.1	7.5±0.5	3.3±0.1	7.7±0.6	6.8±0.2	8.7±0.8	5.4±0.3	7.1±0.4	5.8±0.0	11.3±0.3
18:2n-6	3.7±0.2	5.8±0.8	-	-	-	-	-	-	-	-
18:3n-3	-	-	-	5.1±0.3	-	-	-	-	-	-
18:3n-6	-	-	-	-	-	-	-	-	-	-
18:4n-3	-	-	-	-	-	-	-	-	-	-
20:2n-6	-	-	-	-	291±3.4	413±15.5	-	-	-	-
20:3n-6	-	-	-	-	-	-	-	-	-	-
20:3n-3	-	-	-	-	-	-	26.7±2.8	110±4.2	-	-
20:4n-3	-	-	-	-	-	-	-	1.6±0.1	-	-
22:1n-9	158.3±7.9	130.3±7.6	142.2±4.4	111±14.7	145.4±18.2	155.6±8.6	111.4±12.8	112.4±13.2	159.1±3.7	157±4.4
24:5n-3	-	-	-	-	-	-	-	-	136.1±5.4	190±4.6
24:6n-3	-	-	-	-	-	-	-	-	-	1.3±0.1

[a]Desaturation products were quantitatively computed as µg of fatty acids of total medium per culture (10 ml). Mean ± SEM, n ≥ 3 (independent experiment).

Supplementary Table: Primers used in the study

Target gene	Primer name	Sequence (5'-->3')
FADS2 partial cds	PSfads2-F	GTCTACACCTGGGAAGAGGTCC
	PSfads2-R	GAGGTGTCCACTGAACCAGTCG
FADS2 3'end	QtT (cDNA synthesis)	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCT$_{17}$
	Fads3'raceGSP1-F	ATGAATCATCTGCCGATGGACATCG
	Qo-R	CCAGTGAGCAGAGTGACG
	Fads3'raceGSP2-F (nested PCR)	TCTCCCTGTTGGTGTTATAG
	Qi-R (nested PCR)	GAGGACTCGAGCTCAAGC
FADS2 5'end	Fads5'raceRT-R (cDNA synthesis)	GGGCTTCAGAAACTTTCGC
	QtT-F	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCT$_{17}$
	Qo-F	CCAGTGAGCAGAGTGACG
	Fads5'raceGSP1-R	TTTGTTCCGGTCCTGGCTTGG
	Qi-F (nested PCR)	GAGGACTCGAGCTCAAGC
	Fads5'raceGSP2-R (nested PCR)	GCATCCTCTCCAGCGTAGTGG
FADS2 full length sequence	Fads-F	AGGTGGATCAAGATCAAGGCCAG
	Fads-R	CGATTTATGATGCAGAGGAGGAG
FADS2 ORF cloning	FadsEcoRI-F	CCGGAATTCAGGATGGGAGGTGGAGGCCAG
	FadsXhoI-R	CCGCTCGAGTCATTTATGGAGATATGCATCGAGC
FADS2 ORF sequencing	pYES2-F	CTGGGGTAATTAATCAGCGAAGCG
	pYES2-R	CGTGACATAACTAATTACATGATGC

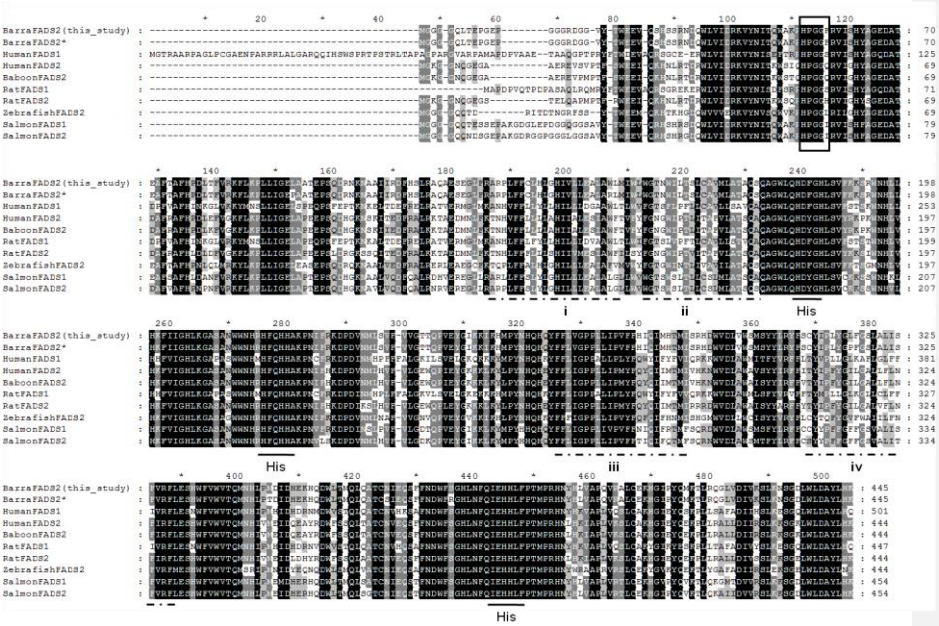
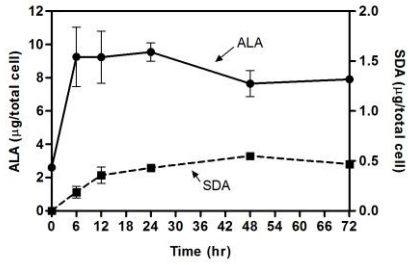


Fig. 1

a


ALA
SDA
ALA (μg/total cell)
SDA (μg/total cell)
Time (hr)

b

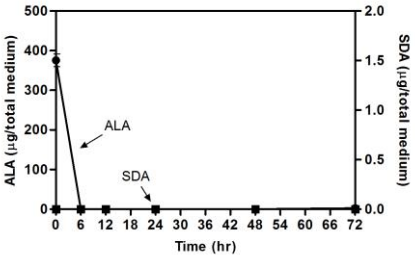
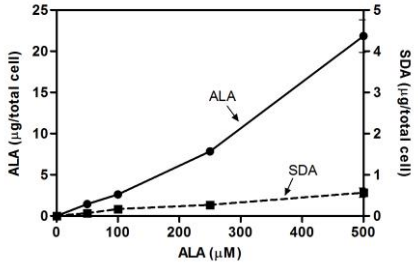

ALA
SDA
ALA (μg/total medium)
SDA (μg/total medium)
Time (hr)

Fig. 2

a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SDA (μg/total cell)
ALA
SDA
ALA (μM)

b

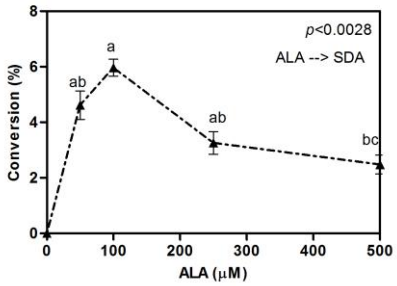
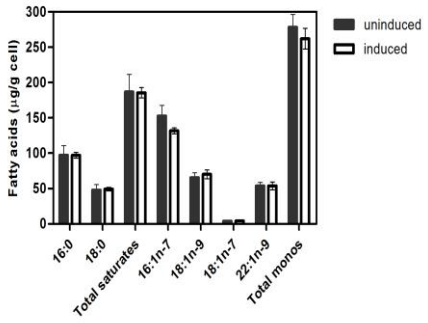

p<0.0028
ALA --> SDA
Conversion (%)
a
ab
ab
bc
ALA (μM)

Fig. 3


300
250
200
150
100
50
0
Fatty acids (µg/g cell)
uninduced
induced
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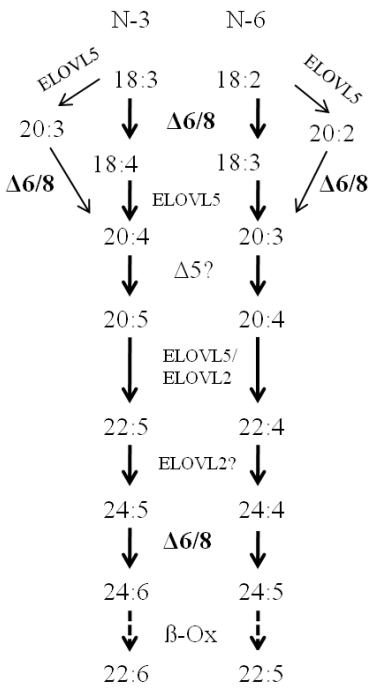
18:0
Total saturates
16:1n-7
18:1n-9
18:1n-7
22:1n-9
Total mones

Fig. 4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ELOVL5
20:4
20:3
Δ5?
20:5
20:4
ELOVL5/
ELOVL2
22:5
22:4
ELOVL2?
24:5
24:4
Δ6/8
24:6
24:5
ß-Ox
22:6
22:5

Fig. 5