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An alternative n-3 fatty acid elongation pathway utilising 18:3n-3 in barramundi (*Lates calcarifer*)

Wei-Chun Tu [a], Beverly S. Mühlhäusler [a], Michael J. James [b], David A. J. Stone [c] and Robert A. Gibson [a*]

[a] FOODplus Research Centre, School of Agriculture, Food and Wine, University of Adelaide, South Australia, Australia

[b] Rheumatology Unit, Royal Adelaide Hospital, South Australia, Australia

[c] South Australian Research and Development Institute, South Australia, Australia

[*]To whom correspondence should be addressed:

Prof Robert A. Gibson

FOODplus Research Centre, School of Agriculture, Food and Wine,

Waite Campus, University of Adelaide, South Australia, 5064, Australia

Phone +618 8303 4333, Fax +618 8303 7135, e-mail: robert.gibson@adelaide.edu.au

ABSTRACT

Desaturase and elongase are two key enzyme categories in the long-chain polyunsaturated fatty acid (LCPUFA) pathway that convert dietary α-linolenic acid (18:3n-3) to docosahexaenoic acid (22:6n-3). The Δ6 desaturase is considered as rate limiting in the conversion. In a previous study in barramundi we demonstrated that the desaturase had a low Δ6 activity but noted that the enzyme also possessed Δ8 ability that utilised 20-carbon fatty acids. This observation suggests that an alternative pathway may exist in the barramundi via elongases to form 20-carbon metabolites from 18:3n-3 to 20:3n-3 and then Δ6/8 desaturase to 20:4n-3. Cloning of the barramundi elongation of very long-chain fatty acid gene (ELOVL) and heterologous expression of the corresponding elongase were performed to examine activity with regard to time course, substrate concentration and substrate preference. Results revealed that the barramundi elongase showed a broad range of substrate specificity including 18-carbon PUFA (including 18:3n-3 and 18:2n-6), 20- and 22-carbon LCPUFA, with greater activity towards omega-3 (n-3) than n-6 fatty acids. The findings from this study provide molecular evidence for an alternative n-3 fatty acid elongation pathway utilising 18:3n-3 in barramundi.

KEYWORDS: α-linolenic acid (18:3n-3), elongase, ELOVL, heterologous expression, LCPUFA metabolism

INTRODUCTION

Dietary 18-carbon polyunsaturated fatty acids (PUFA) such as α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6) are the essential precursors for biosynthesis of long-chain (LC) PUFA. The conventional n-3 LCPUFA pathway involves a Δ6 desaturation of 18:3n-3 to 18:4n-3, followed by elongation to 20:4n-3 and then Δ5 desaturation to eicosapentaenoic acid (20:5n-3) and two consecutive elongations followed by a second Δ6 desaturation and a β-oxidation leading to docosahexaenoic acid (22:6n-3) [1]. Seven fatty acid elongases have been identified from mouse [2,3], rat [4], and human genomes [5,6] and are termed elongation of very long-chain fatty acid gene (ELOVL) subtypes 1−7. Among the mammalian elongase family, ELOVL5 uses a broad range of substrates including 16-carbon monounsaturated fatty acids, 18-carbon PUFA and 20-carbon LCPUFA [4,7]. ELOVL2 is reported to preferentially elongate 22-carbon LCPUFA such as docosapentaenoic acid (22:5n-3) [8]. Together, ELOVL5 and ELOVL2 are utilised in endogenous PUFA synthesis to convert dietary essential fatty acid precursors 18:2n-6 and 18:3n-3 to 24-carbon LCPUFA and eventually to generate 22:6n-3 [9,10].

Beneficial effects of n-3 LCPUFA particularly 22:6n-3 and 20:5n-3 in human nutrition have been widely reported [11,12] and fish is recognised as a major source of these n-3 LCPUFA in human diets [13]. Although aquaculture is widespread, the n-3 content of farmed fish is maintained by providing them with a feed containing fishmeal and/or fish oil [14]. However, the supply of wild fish for preparing these ingredients is limited, and the sustainability of aquaculture will ultimately rely on the ability of producers to make greater use of plant n-3 fatty acids. It is known that freshwater fish species are capable of

converting dietary 18:3n-3 into 20:5n-3 and 22:6n-3 via the desaturation and elongation pathway described above. Marine species, however, have a limited ability to convert n-3 PUFA to n-3 LCPUFA, and it has been suggested that this may be due to the deficiency or impairment of their desaturation/elongation enzyme system [15].

Barramundi (*Lates calcarifer*) is diadromous and thus is 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. We have previously demonstrated that barramundi desaturase has dual Δ6/Δ8 activities which could use 18-carbon and 20-carbon fatty acids [16]. Interestingly, the barramundi Δ6/Δ8 dual function enzyme showed a higher preference for Δ8 than Δ6 activity when encountering the substrate 20:3n-3 and this raises the potential for synthesis of n-3 LCPUFA from 18:3n-3 via a pathway that bypasses the initial Δ6 desaturase step in this species [16].

The aim of the present study was to determine to what extent barramundi elongase has the capacity to synthesize n-3 LCPUFA from 18- and 20-carbon substrates, and in particular, whether the barramundi has the capacity to use 18:3n-3 and 18:2n-6 to form their 20-carbon elongation products for further processing by the Δ8 desaturase. 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 diets in barramundi aquaculture while maintaining the LCPUFA status of the farmed fish.

MATERIALS AND METHODS

Chemicals

Organic solvents used in this study were all analytical grade from Ajax Finechem Pty Ltd (Auckland, New Zealand) or Chem-Supply (SA, Australia). Fatty acids 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 (all > 98-99% purity) were purchased from Cayman Chemicals (MI, USA). Fatty acid 17:0 was from Nu-Chek Prep, Inc (MN, USA) and 24:5n-3 was purchased from Larodan Fine Chemicals (Malmö, Sweden). Other chemicals and reagents were purchased from Sigma-Aldrich (MO, USA) unless specified otherwise.

Barramundi

The protocol was approved by the Animal Ethics Committee, University of Adelaide (Ethics number S-28-08). Juvenile barramundi (*Lates calcarifer*) were obtained from a commercial supplier (W. B. A. Hatcheries, SA, Australia).

3'/5' rapid amplification of cDNA end (RACE)

RNA was isolated from barramundi liver using a Qiagen RNeasy kit (Qiagen, VIC, Australia) following the protocol provided by the manufacturer with the tissue initially disrupted using a Tissue Lyser (Mixer MM 300, Haan, Germany). All primers used in

this study were summarized in **supplementary data 1**. Barramundi ELOVL gene was obtained by a one-step reverse transcription polymerase chain reaction (RT-PCR) with primers designed from the conserved regions of the same genes of other species. The RACE protocol was modified from methods of Frohman [17] to obtain a full-length of the barramundi elongase gene. The cDNA for 3' RACE was reverse transcribed from RNA using a hybrid primer (QtTVN) and reverse transcriptase (Omniscript RT, Qiagen) to generate a 3'end partial ELOVL sequences. PCR amplification was performed using Qo-R and Elovl3'raceGSP1-F primers with HotStar HiFidelity Polymerase (Qiagen).

The nested PCR was performed using Elovl3'raceGSP2-F and Qi-R primers. To generate 5'end partial cDNA clones, reverse transcription was carried out using RNA with the Elovl5'raceRT-R primer to synthesize first strand products. A polyA tail was appended to the cDNA template using terminal deoxynucleotidyltransferase (TdT) (Invitrogen, VIC, Australia) and dATP (Promega, NSW, Australia). PCR amplification was performed using the QtT-F primer to form the second strand of cDNA and Qo-F primer with the Elovl5'raceGSP1-R used for reverse transcription. A nested PCR was carried out using Elovl5'raceGSP2-R and Qi-F primers. Both 3' and 5'RACE products of the ELOVL were sequenced and aligned with the partial gene fragments to identify the overlapping regions.

Cloning and sequence analysis

Elovl-F and Elovl-R primers were designed for determining the full nucleotide sequence of the barramundi ELOVL, and ElovlEcoRI-F and ElovlXhoI-R were used to amplify the PCR product containing the putative ELOVL open reading frame (ORF). The putative barramundi ELOVL ORF was purified, restriction digested and inserted into EcoRI and XhoI sites digested pYES2 vector (Invitrogen) to yield the constructed plasmid pYES2/BarraELOVL. The barramundi elongase coding region was cloned and then sequenced (GenBank ID: GU047382) by pYES2-F and pYES2-R primers. The HotStar HiFidelity Polymerase Kit (Qiagen) with proofreading function was used throughout the cloning processes to minimize potential PCR errors. Sequence results indicated that the putative barramundi elongase gene included an ORF of 885 bp nucleotides specifying a protein of 294 amino acids. Parallel alignment of barramundi putative elongase (GenBank ID: GU047382, this study) with another barramundi elongase sequence (GQ214180) reported previously [18] indicated that the two barramundi ELOVL were 100% identical in peptide sequence.

Yeast transformation

The pYES2/BarraELOVL plasmid was then transformed into *Saccharomyces cerevisiae* (INVSc1 yeast strain, Invitrogen) using *S. c.* EasyComp^{TM} Transformation Kit (Invitrogen). Transformed yeast cells 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.

147

148 **Heterologous expression and incubation of fatty acids**

149 An INVSc1 transformant colony containing pYES2/BarraELOVL construct was
150 inoculated into SC^{-U} medium containing 2% glucose. The cells were grown overnight at
151 27°C in an orbital incubator with vigorous shaking at 130 rpm. Expression of barramundi
152 elongase was induced by transferring log-phase yeast cells (OD_{600nm} = 0.4) into SC^{-U}
153 medium containing 2% galactose and 0.25% tergitol. Cultures were supplemented with a
154 single fatty acid substrate from: 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6,
155 with concentrations of 250 μM for time course experiments, 0−500 μM for dose-response
156 studies and 500 μM for a parallel comparison of substrate specificity. Yeast cells
157 transformed with pYES2/BarraELOVL plasmid without galactose induction were used as
158 negative controls. After 6, 12, 24, 48 and 72 hr (time course study) or 24hr (dose-
159 response and substrate specificity studies) incubation, yeast cells and culture media from
160 individual culture with three independent replicates were harvested and total lipids were
161 extracted and analysed as described below.
162

163 **Lipid extraction, methylation and gas chromatographic (GC) analysis**

164 All yeast cells from 10 ml culture from each treatment were harvested. One ml of cell-
165 free medium from each incubation was collected. Fatty acid 17:0 was added into each
166 extraction tube as an internal standard. Total lipids were extracted from the samples by
167 vigorous vortexing in chloroform-isopropanol (2:1, v/v) following a modified protocol of

Bligh and Dyer [19]. The resulting chloroform phase was evaporated to dryness under nitrogen gas, 1% sulphuric acid in methanol was added and transmethylation was performed. The fatty acid methyl esters were extracted with *n*-heptane, separated and quantified by GC as described previously [16].

Statistical analysis

A one-way analysis of variance (ANOVA) followed by Tukey-HSD test was used if P value less than 0.05 and the data followed Gaussian distributions. Kruskal-Wallis test with Dunn's multiple comparison test were applied for non-parametric analyses if data did not pass the normality test. Unpaired t-test was used to identify a 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).

RESULTS

Elongation activity of the barramundi putative ELOVL

Time course

18:4n-3 and 20:5n-3 were used to examine the barramundi elongase expression activity because the peptide sequence alignment of the barramundi ELOVL with ELOVL2 and ELOVL5 in human, zebra fish and Atlantic salmon indicates a higher degree of homology with ELOVL5 (data not shown), and both 18:4n-3 and its downstream product, 20:5n-3, are considered the main substrates for LCPUFA conversion by ELOVL5.

Following the addition of 18:4n-3 to transformed yeast cells expressing the barramundi ELOVL gene, the first elongation product, 20:4n-3, peaked at 6 hr but decreased to a lower constant level at all other time points (**Fig. 1 A**). In contrast, there was a linear increase with time of the next elongation product, 22:4n-3, in the yeast cells over the entire 72 hr of the incubation (**Fig. 1 A**). The concentration of extracellular elongation products (i.e. those in the culture medium) was also examined and a similar time course patterns were seen for 20:4n-3 and 22:4n-3, indicating secretion or diffusion of these fatty acids from yeast lipids into the medium (**Fig. 1 B**). There was also a significant decrease of 18:4n-3 in the medium within 6 hr, indicating the substrate was taken up by cells (**Fig. 1 B**). When the total mass in yeast cell + medium is calculated, it is clear that within 6 hr, less than 5% of the original 18:4 n-3 substrate was left in the culture and around 12% was converted to 20:4n-3 and 22:4n-3 (**Fig. 1 C**). The substrate disappearance was also observed in the cultures with 20-carbon fatty acids. Approximately 25% of the 20:5n-3 substrate was left in the culture and 10% of was converted to 22:5n-3 within 6 hr. However, 24:5n-3 was not detectable until 12 hr (**Fig. 2 E and F**). Elongation of 20:5n-3 to 22:5n-3 showed a similar time course to that observed with 18:4n-3, except that there was very limited conversion to the second elongation product, 24:5n-3 (**Fig. 1 D-F**).

Effect of substrate concentration

With increasing concentrations of 18:4n-3 during 24 hr incubation, the production of 20:4n-3 and 22:4n-3 in yeast cells increased linearly up to 500 μM of 18:4n-3 (**Fig. 2 A**) and a significant amount of 22:4n-3 was observed in the medium (**Fig. 2 B**). When the total mass in yeast cell + medium was calculated, it is clear that both elongation reactions, namely conversion of 18:4n-3 to 20:4n-3 and 20:4n-3 to 22:4n-3, effectively

212 preceded in a dose–dependent manner, and more than 95% of 18:4n-3 at 500 μM was
213 elongated to 22:4n-3 (**Fig. 2 C**). With the addition of 20:5n-3 up to 500 μM there was an
214 increase in the concentration of the first elongation product, 22:5n-3, in both cells and
215 incubation medium but only low amounts of the second elongation product, 24:5n-3, in
216 either the yeast or the medium (**Figs. 2 D** and **E**) suggesting limited ability of the
217 transformed yeast cells to produce 24:5n-3. When summing the fatty acids in both the
218 yeast cells and the medium for calculating the conversion rates, more than 90% of 20:5n-
219 3 at 500 μM was elongated into 22:5n-3 (**Fig. 2 F**). The ability of the transformed yeast
220 cells to elongate 18:3n-3 to 20:3n-3, the potential bypass pathway of the LCPUFA
221 conversion, is shown in **Fig. 2 G-I**. After the addition of 18:3n-3 to the transformed yeast
222 cells, the elongation products 20:3n-3 plus a small amount of 22:3n-3 were detected in
223 the yeast cells (**Fig. 2 G**) and the culture medium (**Fig. 2 H**). When the amount of all
224 substrates and products in the yeast cells and medium were calculated, at 500 μM of
225 18:3n-3, the conversion efficiency of 18:3n-3 to 20:3n-3 was above 60% (**Fig. 2 I**).

226 ***Substrate preferences***

227 We compared substrate preferences for a range of n-3 fatty acids and their n-6
228 homologues in the elongation pathway of LCPUFA. All fatty acid substrates were
229 supplemented at 500 μM into yeast cultures and the cells incubated for 24 hr. When
230 summing the fatty acids in both the yeast cells and the medium into account for
231 calculating the conversion rates, there was a preference for n-3 over n-6 18-carbon fatty
232 acids i.e. 18:4n-3 (97%) was preferred over 18:3n-6 (79%), and 18:3n-3 (62%) over
233 18:2n-6 (23%) (**Table 1**). The n-3/n-6 preference was also significant ($P = 0.02$) between
234 20:5n-3 and 20:4n-6.

The effect of induction of the barramundi ELOVL on endogenous yeast fatty acids, which are mainly saturated and monounsaturated fatty acids, was also examined. Induction of the enzyme increased the amounts of 18:1n-7, 18:1n-9 and 20:1n-9 in the cells significantly but there was no effect on endogenous saturated fatty acids (data not shown).

DISCUSSION

Using yeast as a heterologous expression model to examine the substrate specificity of the recombinant barramundi elongase, we observed that the barramundi ELOVL showed efficient activity at elongating the homologous pairs 18:4n-3 and 18:3n-6 as well as 18:3n-3 and 18:2n-6 to their 20-carbon metabolites, albeit with lower efficiencies. The same pattern of elongation efficiency has been reported in other species [4,5,20]. The recombinant barramundi ELOVL also showed high efficiency for utilising the 20-carbon substrates 20:5n-3 and 20:4n-6 to produce 22-carbon products. In contrast, the recombinant enzyme only showed weak activity towards 22-carbon LCPUFA, indicating a high degree of activity with ELOVL5. The preference for n-3 over n-6 fatty acids was observed for both the 18- and 20-carbon substrates.

The preference for n-3 fatty acids differs from a previous report of barramundi ELOVL activity, which found a preference for n-6 18-carbon PUFA (18:3n-6 over 18:4n-3) [18]. However the preference for n-3 over n-6 fatty acids was observed for the 20-carbon LCPUFA (20:5n-3 over 20:4n-6) by Mohd-Yusof *et al* [18] which is similar to our

findings. The peptide sequences were 100% identical between our ELOVL and that reported by Mohd-Yusof *et al* [18] but they employed a 72 hr incubation period (no detail of the concentration of substrates added). In addition, they did not perform yeast cell + medium mass balance calculations and did not examine 18:3n-3 and 18:2n-6 substrates as we have done, thus making a sensible comparison between the two studies difficult.

Our results suggest that a potential bypass of the Δ6 desaturation involving an elongation of 18-carbon PUFA followed by a Δ8 desaturation, rather than the conventional Δ6 desaturation followed by an elongation, is possible. In this model, the elongation products 20:3n-3 and 20:2n-6 from 18:3n-3 and 18:2n-6, respectively, may be important in the synthesis of 22-carbon LCPUFA if the rate limiting Δ6 desaturation step is appreciably slow [21]. The converted 20:4n-3 and 20:3n-6 (desaturation products of 20:3n-3 and 20:2n-6) could be further utilised by a Δ5 desaturation and the usual pathway. We have observed that fatty acyl desaturase (FADS) from barramundi has dual Δ6/Δ8 desaturase activity when the substrates 18:3n-3 and 20:3n-3 or 18:2n-6 and 20:2n-6 were available [16]. Thus, dietary 18:3n-3 and 18:2n-6 can be elongated by barramundi ELOVL and then desaturated by FADS2 to 20:4n-3 and 20:3n-6. The LCPUFA 20:4n-3 and 20:3n-6 are immediate precursors of 20:5n-3 and 20:4n-6, respectively following the action of a Δ5 desaturase. However, it remains unclear whether the Δ5 desaturase actually exist and function in barramundi [16]. **Fig. 3** illustrates the alternative pathway of ELOVL activity for LCPUFA conversion. Thus, the LCPUFA conversion from 18:3n-3 and 18:2n-6 precursors in barramundi can be processed through either (1) a Δ6 desaturation pathway to form 18:4n-3 and 18:3n-6 and then through an elongation reaction to form the

281 downstream products 20:4n-3 and 20:3n-6 or (2) an initial elongation step to form 20:3n-
282 3 and 20:2n-6 and then followed with a Δ8 desaturation to form 20:4n-3 and 20:3n-6
283 (**Fig. 3**).
284
285 Elongation of 18:3n-3 and 18:2n-6 or 18:4n-3 and 18:3n-6 not only produces 20-carbon
286 metabolites for subsequent Δ5 desaturation but it also could generate some 22-carbon
287 products, i.e. 22:3n-3 and 22:2n-6, or 22:4n-6 and 22:3n-6. These 22-carbon fatty acids
288 are considered as 'dead-end' products because they are not part of the classic LCPUFA
289 synthesis pathway (**Fig. 3**). Whether these fatty acids are produced, and if they are stored
290 in the tissue or can be utilised by other enzymes remains to be elucidated.
291
292 From the time course experiments, we observed that most 18- and 20-carbon fatty acids
293 disappeared in the culture and 18-carbon fatty acid substrate appeared to be lost more
294 than 20-carbon substrate. For example, there was 5 times more 20-carbon substrate (25%
295 of original substrate) left in the culture by 6 hr incubation than 18-carbon substrate (<
296 5%). Only a small proportion of the exogenous fatty acids followed the biosynthetic
297 pathway to be elongated to their longer chain metabolites, suggesting fatty acids are
298 possibly oxidised by the yeast cells to produce energy [16,22]. Further, we have
299 previously demonstrated that desaturation products also appear in the culture medium
300 [16], emphasising the importance of measuring and taking into account any elongation
301 products present in the medium in any calculation of enzyme activity.
302

In summary, we have examined a barramundi ELOVL gene which yielded a protein that exhibited the greatest elongase activity towards 20-carbon LCPUFA, and generally showed greater conversion of n-3 compared with n-6 substrates. Most importantly, the elongase has a capacity for the conversion of 18:3n-3 and 18:2n-6 to their 20-carbon products, suggesting an alternative pathway for LCPUFA conversion in barramundi. Bypassing the first (and limiting) Δ6 desaturation step has the potential to improve downstream LCPUFA conversion. However, whether this alternate pathway would be of benefit for the aquaculture industry remains to be examined.

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FIGURE LEGENDS

Fig. 1. Time course of changes in fatty acid levels of elongation product after expressing barramundi elongase with addition of 18:4n-3 or 20:5n-3 fatty acid substrate at 250 μM in transformed yeast cultures. Fatty acid levels in cells (A), in remaining cell-free medium from yeast culture (B) and fatty acid in cells and medium were both taken into account (C) after addition of 18:4n-3. Fatty acid levels in cells (D), in remaining cell-free medium from yeast culture (E) and fatty acids in cells and medium were both taken into account (F) after addition of 20:5n-3. Mean ± SEM, n=3.

Fig. 2. Dose-response of 18:4n-3, 20:5n-3 or 18:3n-3 at 0−500 μM for 24 hr on barramundi elongase activity and production of n-3 LCPUFA. Effect of 18:4n-3 on biosynthesis of 20:4n-3 and 22:4n-3 in cells (A), in remaining cell-free medium from yeast culture (B) and fatty acids in cells and medium were both taken into account (C). Effect of 20:5n-3 on synthesis of 22:5n-3 and 24:5n-3 in cells (D), in remaining cell-free medium from yeast culture (E) and fatty acids in cells and medium were both taken into account (F). Effect of 18:3n-3 on synthesis of 20:3n-3 and 22:3n-3 in cells (G), in remaining cell-free medium from yeast culture (H) and fatty acids in cells and medium were both taken into account (I). Elongation products were quantitatively computed as μg of fatty acids per total cell, medium or total cell + medium. Mean ± SEM, n=3.

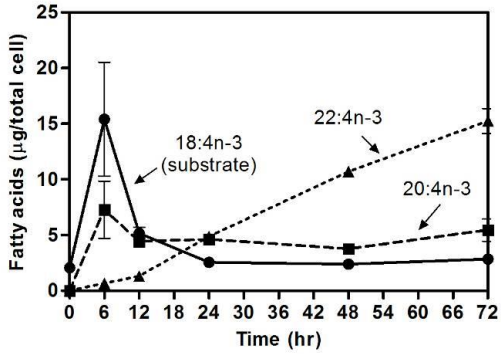
Fig. 3. A postulated bypass (dashed line) of LCPUFA biosynthesis utilising an elongation pathway in barramundi. Bold font indicates n-3 fatty acids and non-bold font indicates n-6 fatty acids.

Table 1. Substrate specificity and enzyme activity

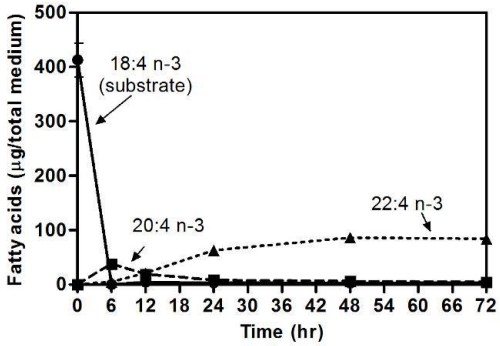
Fatty acid substrate[a]	Elongation product	Conversion (%)		Enzyme activity
		Cells only[b]	Cells + Medium[c]	
n-3 PUFA				
18:3n-3	20:3n-3	31.2±0.8	50.5±1.8	Elongase5
	22:3n-3	1.7±0.1	11.3±0.6	
18:4n-3	20:4n-3	38.3±0.9	20.4±0.8	Elongase5
	22:4n-3	38.6±2.1	76.2±1.3	
20:5n-3	22:5n-3	72.1±2.3	91.9±0.5	Elongase5/2
	24:5n-3	2.1±0.2	4.9±0.5	
n-6 PUFA				
18:2n-6	20:2n-6	11.2±1.2	23.2±2.2	Elongase5
	22:2n-6	n.d.[d]	n.d.	
18:3n-6	20:3n-6	45.7±0.5	43.1±0.7	Elongase5
	22:3n-6	7.3±0.4	35.4±0.6	
20:4n-6	22:4n-6	46.0±1.6	91.5±0.3	Elongase5/2
	24:4n-6	n.d.	n.d.	

[a]All fatty acids were supplemented 500 μM into yeast culture medium and the cells cultured for 24 hr.
[b]Fatty acid in yeast cells only were used for calculating the conversion (%). Data are means ± SEM of $n \geq 3$. Conversion (%) = [cellular product / (cellular substrate + cellular product) × 100].
[c]Elongated fatty acid products in medium were also 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].
[d]n.d. = not detected.

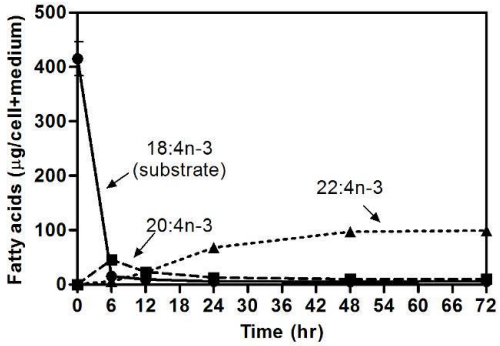
A


Fatty acids (μg/total cell)
Time (hr)
18:4n-3
(substrate)
22:4n-3
20:4n-3

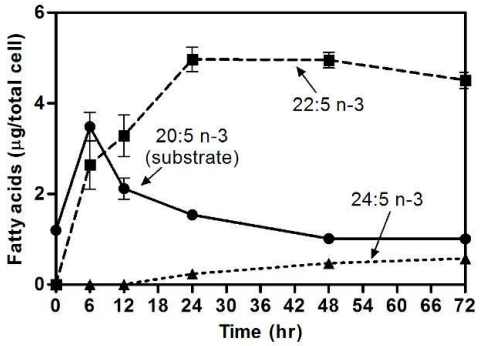
B


Fatty acids (μg/total medium)
Time (hr)
18:4 n-3
(substrate)
20:4 n-3
22:4 n-3

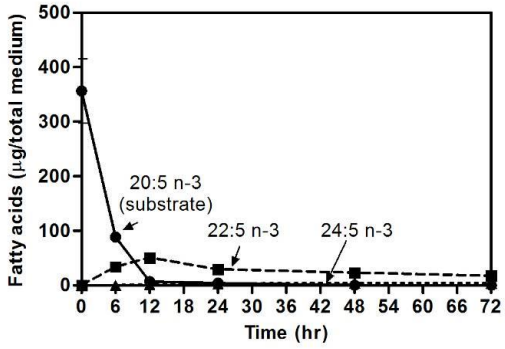
C


Fatty acids (μg/cell+medium)
Time (hr)
18:4n-3
(substrate)
20:4n-3
22:4n-3

D


Fatty acids (μg/total cell)
Time (hr)
22:5 n-3
20:5 n-3
(substrate)
24:5 n-3

E


Fatty acids (μg/total medium)
500
400
300
200
100
0
20:5 n-3
(substrate)
22:5 n-3
24:5 n-3
0 6 12 18 24 30 36 42 48 54 60 66 72
Time (hr)

F

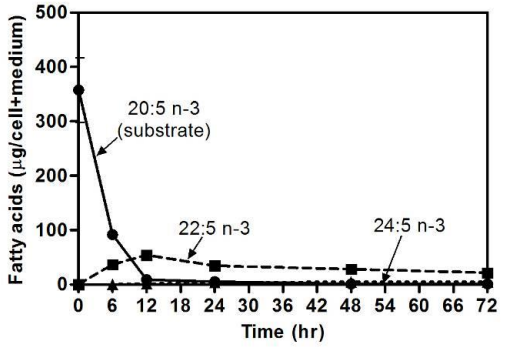
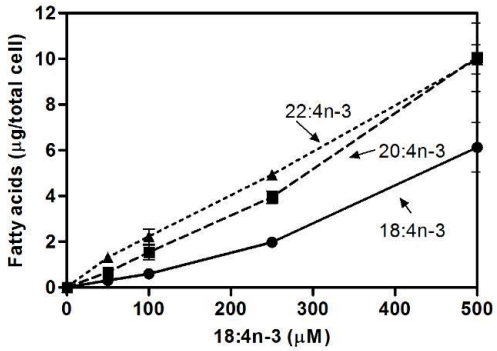
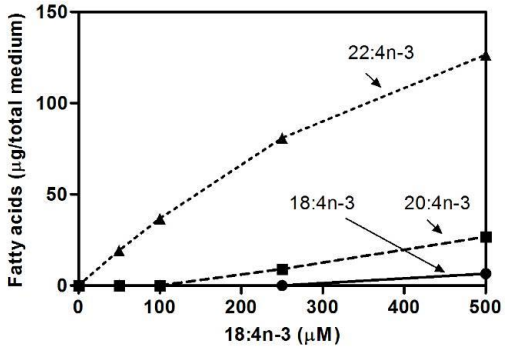

Fatty acids (μg/cell+medium)
500
400
300
200
100
0
20:5 n-3
(substrate)
22:5 n-3
24:5 n-3
0 6 12 18 24 30 36 42 48 54 60 66 72
Time (hr)

Fig 1.

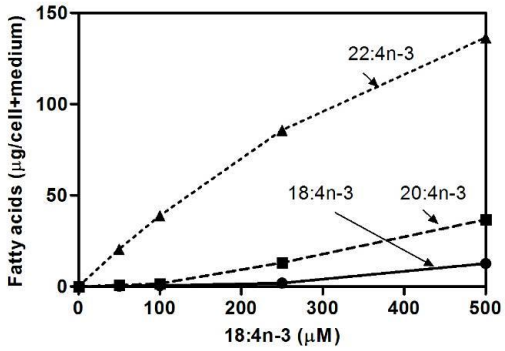
A


Fatty acids (μg/total cell)
12
10
8
6
4
2
0
0
100
200
300
400
500
18:4n-3 (μM)
22:4n-3
20:4n-3
18:4n-3

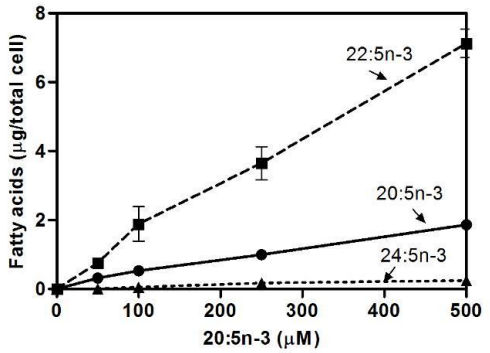
B


Fatty acids (μg/total medium)
150
100
50
0
0
100
200
300
400
500
18:4n-3 (μM)
22:4n-3
18:4n-3
20:4n-3

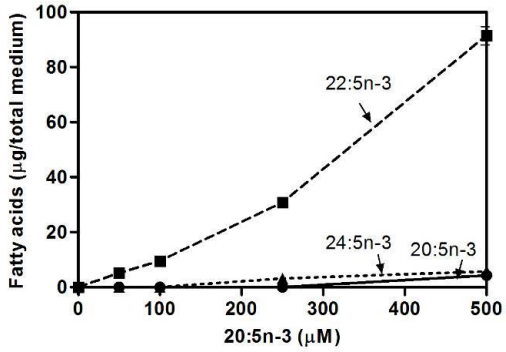
C


Fatty acids (μg/cell+medium)
150
100
50
0
0
100
200
300
400
500
18:4n-3 (μM)
22:4n-3
18:4n-3
20:4n-3

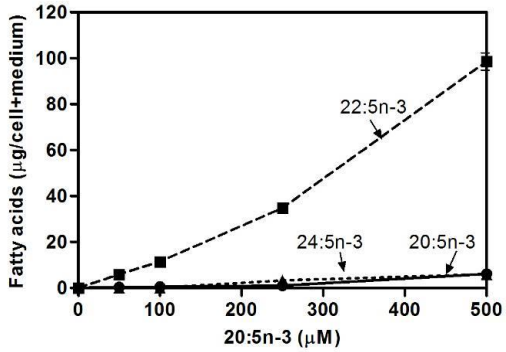
D


Fatty acids (μg/total cell)
22:5n-3
20:5n-3
24:5n-3
20:5n-3 (μM)

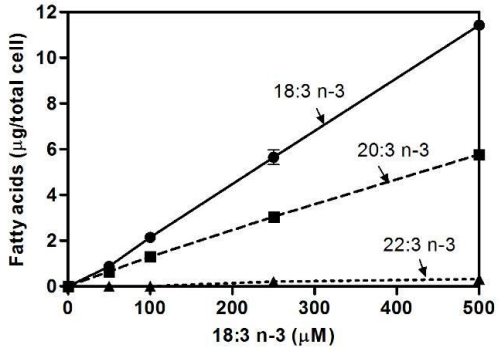
E


Fatty acids (μg/total medium)
22:5n-3
24:5n-3
20:5n-3
20:5n-3 (μM)

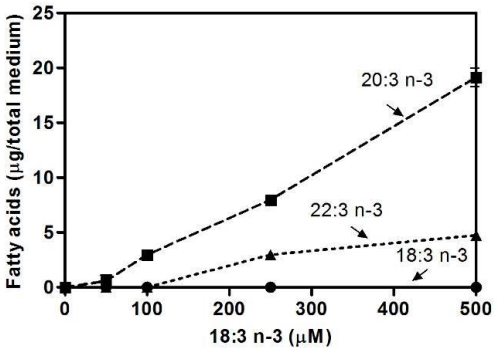
F


Fatty acids (μg/cell+medium)
22:5n-3
24:5n-3
20:5n-3
20:5n-3 (μM)

G


Fatty acids (μg/total cell)
18:3 n-3
20:3 n-3
22:3 n-3
18:3 n-3 (μM)

H


Fatty acids (μg/total medium)
25
20
15
10
5
0
0
100
200
300
400
500
18:3 n-3 (μM)
20:3 n-3
22:3 n-3
18:3 n-3

I

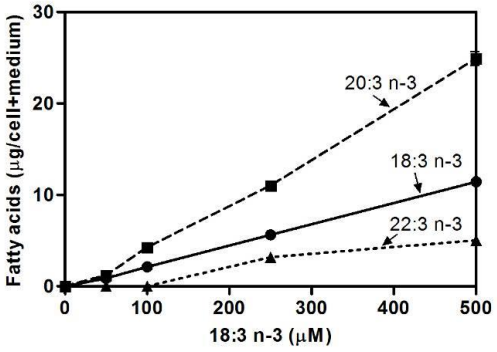

Fatty acids (μg/cell+medium)
30
20
10
0
0
100
200
300
400
500
18:3 n-3 (μM)
20:3 n-3
18:3 n-3
22:3 n-3

Fig 2.

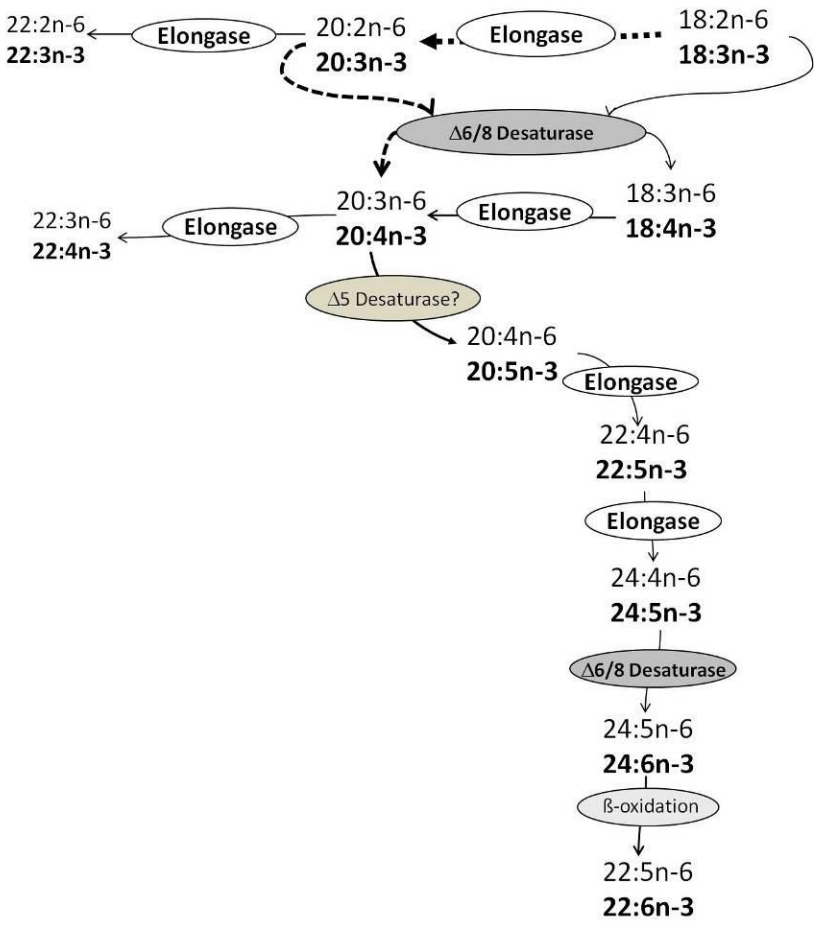

22:2n-6
22:3n-3
Elongase
20:2n-6
20:3n-3
Elongase
18:2n-6
18:3n-3
Δ6/8 Desaturase
20:3n-6
20:4n-3
Elongase
18:3n-6
18:4n-3
22:3n-6
22:4n-3
Elongase
Δ5 Desaturase?
20:4n-6
20:5n-3
Elongase
22:4n-6
22:5n-3
Elongase
24:4n-6
24:5n-3
Δ6/8 Desaturase
24:5n-6
24:6n-3
ß-oxidation
22:5n-6
22:6n-3

Fig 3.

Supplementary data 1. Primers used in the study

Target gene	Primer name	Sequence (5'->3')
ELOVL partial cds	PSelovl-F	GGATGGGGCCCAAGTACATG
	PSelovl-R	GTCTGCATGTAGAAGTTTGAG
ELOVL 3'end	QtTVN (cDNA synthesis)	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCT$_{17}$VN
	Elovl3'raceGSP1-F	GGTCTACAATCTGGGCCTC
	Qo-R	CCAGTGAGCAGAGTGACG
	Elovl3'raceGSP2-F (nested PCR)	GACCCAGACAATGTGTGCAGTC
	Qi-R (nested PCR)	GAGGACTCGAGCTCAAGC
ELOVL 5'end	Elovl5'raceRT-R (cDNA synthesis)	CATGTAGAAGGACAAGAGCG
	QtT-F	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCT$_{17}$
	Qo-F	CCAGTGAGCAGAGTGACG
	Elovl5'raceGSP1-R	CCAGATTGTAGAGCACCAGGAG
	Qi-F (nested PCR)	GAGGACTCGAGCTCAAGC
	Elovl5'raceGSP2-R (nested PCR)	GGCTGCCTGTGTTTCATGTAC
ELOVL full length sequence	Elovl-F	CTCTCTCTCCCCCCGCCTC
	Elovl-R	GACCATAGTAAGCACTGTGTTG
ELOVL cloning	ElovlEcoRI-F	CCGGAATTCCAAATGGAGACCTTCAATCATAAAC
	ElovlXhoI-R	CCGCTCGAGATGTCAATCCACCCTCAGTTTC
ELOVL ORF sequencing	pYES2-F	CTGGGGTAATTAATCAGCGAAGCG
	pYES2-R	CGTGACATAACTAATTACATGATGC

343

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