Omni-Akuatika, 13 (2): 73 – 79, 2017
ISSN: 1858-3873 print / 2476-9347 online

Research Article

Δ6-desaturase-Like Encoding Gene Introductionin Catfish (*Clarias gariepinus*)

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Received 1 July 2017; Accepted 7 October 2017; Available online 28 November 2017

ABSTRACT

African catfish (*Clarias gariepinus*) is one of the economically valuable aquaculture fish species in Indonesia. This study aims to acquire a DNA concentration that could generate high viable sperm, and F0 transgenic fish. In addition, the fish fatty acids contents would be also analyzed.The Δ6-desaturase enzyme was involved in highly unsaturated fatty acid biosynthesis. Transgenic catfish was produced by sperm-mediated gene transfer using electroporation method. In this study, as the first step, sperms were electroporated with three different OmΔ6FAD concentration (25, 50, and 100 µg mL^{-1}) to have the highest sperm viability after electroporation (125 V cm^{-1}, pulse frequency 5 times, pulse length 30 millisecond, pulse interval 0.1 second). The highest sperm viability and sperm carrying OmΔ6FAD were obtain at 100 µg mL^{-1}. This concentration was then used to produce F0 transgenic catfish in the second step. Sperm motility, sperm viability, fertilization rate, hatching rate, and larval survival at 14 days after hatching were the same as the controls ($p>0.05$). Genomic DNA was extracted from caudal fin and then used as template to identify transgenic F0 by PCR method using specific primer for OmΔ6FAD gene. The PCR result showed that 53.84% of F0 carried \OmΔ6FAD gene. The result of fatty acid analysis showed that EPA and DHA contents of F0 transgenic fish and non-transgenic fish were similar.

Keywords: *catfish, Δ6-desaturase-like gene, fatty acids, electroporation*

1. Introduction

Long chain unsaturated fatty acids, particularly Eicosapentaenoic acid (EPA; C20: 5n-3) and *Docosahexaenoic*acid (DHA; C22: 6n-3) have important nutrional benefits in humans(Simopoulos, 1991; Lauritzen et al., 2001), and are generally found in both freshwater and marine fish species. Freshwater fish have the ability to synthesize EPA and DHA, since they possess all the enzyme coding genes that are involved infatty acids biosynthesis. Meanwhile, marine fish do not possess fatty acids synthesizing enzymes. However, EPA and DHA levels are higher in marine fish compared to the freshwater one, as the former species selectively accumulate EPA and DHA from natural food.For instance, EPA and DHA contents of Atlantic salmonranged between 13.1-15.2% and 6.6-7.9%, respectively (Blancheta et al., 2005), while those of catfish were just 0.68% and 0.43%, respectively (Gunawan et al., 2014).

Δ6-desaturase isa fatty acid metabolic enzyme that is involved inEPA and DHA biosynthesis. This enzyme uses α-linolenic acid (ALA, C18:3n-3) as a substrate and allows the insertion of a double bond to produce octadecatrienoic acid (OTA, C18: 4n-3). The OTA is converted by elongase to produce eicosatetraenoic acid (ETA, C20: 4n-3), a substrate of Δ5-desaturasion in EPA synthesis. The same Δ6-desaturase also metabolizes the fatty acid C24:5n-3 to C24:6n-3, which is finally retro-converted by peroxisomes via β-oxidation to yield DHA (Sprecher, 2000).

Nowadays, the rapid development of biotechnology facilitated the production of fishery products with desired characteristics. Transgenesis is referred to as a genetic engineering technology that allows the production of fish with improved phenotype through the introduction of encoding genes with the desired characteristics. Electroporation is a genetic engineering method, which can be easily applied to gametes, especially sperm (Sarmasik et al., 2001). Both viability and ability ofsperm to fertilize eggs(post-electroporation)

will strongly determine the number of F0 transgenic fish generated. Factors associated with electroporation that affect the sperm viability include: the electric field (strength), duration and length between shocks as well as the DNA concentration.

The success rate of transgenesismethodthrough the use of the Δ6-desaturase-like metabolic encoding enzyme, which was derived from masou salmon *Oncorhynchus masou* (OmΔ6FAD), on zebra fish *Danio rerio*has been reported in previous research (Alimuddin et al., 2005). Over-expressionof these genes resulted in increments in both EPA and DHA contents in zebrafish (second generation, F2), approximately 75% and 79%, respectively, compared to control. In the present study, OmΔ6FAD gene transfer in catfish is expected to increase the fish ability to synthesize EPA and DHA, so that it could be an easily accessed fatty acids (EPA and DHA) source. This study was aimed to acquire a DNA concentration that could generate high viable sperm, and F0 transgenic fish. In addition, the fish fatty acids contents would be also analyzed.

2. Methods

OmΔ6FAD plasmid purification

The constructed genes used in the present studywas pmBA-OmΔ6FAD plasmid containing the OmΔ6FAD gene from masou salmon *Oncorhynchus Masou* with a β-actin promoter (pmBA) from medaka fish *Oryzias latipes*. The constructed gene multiplication was carried out using the standard procedures developed by Sambrook et al. (1989). *Escherichia coli*bacteria, which containedpmBA-OmΔ6FAD plasmid, was multiplied using liquid culture method, harvested from a solid agar, and then cultured on a liquid medium containing triptone (1.6%), yeast extract (1%), NaCl (0.5%) and ampicillin antibiotics. The mixture was then incubated using a shaker at a speed of 250 rpm (at 37°C) for 16-18 h, and the bacteria (from the culture) was put in a 1.5 ml micro tube, and centrifuged at 12,000 rpm for 30 seconds. The plasmid was extracted using a high-speed *plasmid mini kit* (Geneaid Biotech Ltd., Taiwan) by following the instructionsof the manual of procedures. The initial DNA concentration was determined using a genequant (Pharmacia Biotech genequant)and three DNA concentrated solutions were made for gene transfer i.e. 25, 50 dan 100 µg mL^{-1}.

Gamete collection

Catfish broodstock (males and females) at an average body weight of 650-800 gram were used in the present study. Broodstock were selected based on their level of gonadal maturity and ovulation was induced using Ovaprim. Fish were induced according to their body weight i.e. a single dose of 0.1 ml kg^{-1} for males, and 0.3 ml kg^{-1} females (Gusrina, 2011). After observing a period of 8 hour(post-injection), males were dissected, their testes taken and cut into pieces in order to let the sperm out of the testes bags. Meanwhile, the female were gently stripped in order to get eggs out of their abdominal cavity and placed into a bowl. Sperm was diluted with NaCl (0.9%) at a ratio of 1: 1.

Sperm *electrophoration*

Sperm electroporation was carried out using a Gene Pulser Xcell machine (Biorad, USA). Sperm was diluted using physiological solution (1: 1), and then mixed with the plasmid. In order to reach an optimal electroporation condition, apreliminary research was conducted to determine the PMBP-OmΔ6FAD DNA plasmid concentration, which support motility and provide a high sperm survival rate in order to fertilize eggs. DNA concentration testinvolved three doses i.e. 25, 50, and 100 µg ml^{-1}. Sperm without treatment (STP) was prepared as a tool controller, and electrophoresed sperm without DNA as DNA controller.

Program electroporation based Gusrina's method (2011) conducted on catfish with voltage 125 V cm^{-1}, pulse frequency 5 times, pulse length 30 millisecond, pulse interval 0.1 second. The amount of DNA that was mixed with the sperm was calculated based on the initial DNA concentration. The mentioned sperm solution was then mixed with plasmid DNA and 500 microliters of saline. The electrophoresed solution was finally used to fertilize eggs.

Sperm motility and viability

Electrophoresed sperm quality was measured by determining the sperm motility index. Thus, a drop of sperm was dripped on an object glass using a micropipette and then covered with a glass lid. Distilled water (drop) was dripped at the edge of the covering glass in order to observe sperm movement (after being exposed to distilled water) under a microscope at a magnification of 400 ×. Sperm motility was assessed according to the criteria

based on the number of forward moving sperm (progressive) with a scoring method developed by Guest *et al.* (1976).

The sperms viability (post-electroporation) were observed through Giemsa staining method. Sperm was dripped on an object glass, mixed with a Giemsa solution (de la Cueva et al., 1997) and evenly mingled in order to obtain thin pillowcase preparations. The pillowcase preparations were then dried, and observed under a microscope with a magnification of 400×. Live sperms were marked by their round and pink-colored head, while the dead sperms were characterized by both their irregular and black-colored head. The motility and viability of sperms were analyzed using a one way ANOVA, which was followed by post-hoc Tukey test (p=0.05) using SPSS 16.0 (IBM, USA). OmΔ6FAD gene presence in electrophoresed sperm was confirmed through PCR method. DNA genome isolation was carried out using a *DNA Purification Kit* (Puregene, Minneapolis, USA) according to the manual of procedures. PCR was performed using a set of OmΔ6FAD specific primer i.e. forward Masou-F1 (5'-CCTGCGACCGTGTAGAGAGGG-3') and reverse Masou-R1 (5'-AGAAGGCGAAGGTAGAAAGTCATCGC-3'). This primer was designed based on *Clarias gariepinus* mRNA (Fads2) sequence (GeneBank access no. KU925904.1) and *Oncorhynchus Masou* putative mRNA (GeneBank access no. AB070444.1). The PCR program used in the present study was denaturation at 94°C for 30 seconds, annealing at 62°C and extension at 62°C for 30 seconds, and the number of cycles during the PCR amplification was 35. PCR products separation was done through electrophoresis with an agarose gel concentration of 1% (Vivantis, USA). Sperm carrying the OmΔ6FAD gene was characterized by the presence of DNA bands that were similar to those of the positive control with a band size of 529 bp.

Hatching and fish rearing

Sperm and eggs were mixed at a ratio of 1:1 (male: female) in a plastic container, mixed using chicken feather, and incubated in an aquarium (60 cm × 40 cm × 40 cm) which has been previously filled with water and methylene blue (2-3 drops) to prevent fungus formation on eggs. The aquarium was also equipped with aeration system to provide sufficient oxygen. Unfertilized eggs were easily identified by their appearance, while fertilized eggs were characterized by their translucent yellowish color. The fertilization rate and hatching rate were determined 8 hour, and 24 hour (respectively) post-electrophoresis, meanwhile, larvae survival rate was determined 14 days post-electrophoresis.

Larvae were fed on natural food (artemia) at *ad libitum* from day 2 until day 4 post-hatched, and on worms from day 3 until the end of the rearing period. Fertilization rate, hatching rate, and larvae survival rate were analyzed using a one way ANNOVA, which was followed by post-hoc Tukey test (p = 0.05) using SPSS 16.0 (IBM, USA).

Transgenic fish (F0) *identification*

Early individual transgenic fish (F0) carrying the OmΔ6FAD gene were identified using PCR with a genomic DNA template that has been extracted (from the tail fin of the fish) at the age of 4 weeks(post-hatched). Genomic DNA extraction, primers, PCR amplification, and electrophoresis methodswere similar to the methods used in confirming the presence of the OmΔ6FAD gene in sperm. As an internal control, samples were identified through the PCR method using a set of β-actin universal primers i.e. β-actin forward univ (5'-GACCTCACAGACTACCTCATG-3 ') and reversed β-actin univ (5'-TCATTGCCCA-TGGTCATGACC-3'). Transgenic Fish (F0) carrying the OmΔ6FAD gene were characterized by the presence of DNA bands that were similar to those of the positive control with a band size of 529 bp.

Fatty acids profile analysis

Fatty acids analysis was carried out usinggas chromatography (GC) method. Twelve transgenic fish and seven non-transgenic fish at average body length and weight of 7-8 cm and 50 g, respectively, were used as samples. The samples were prepared as followed : Oil sample (30-40 mg)were placed into a capped tube, then 2 mL of NaOH (0.5M) in methanol were added, and heated in a water bath for 20 minutes. The samples were cooled, then 2 mL of 14% BF3 in methanol were added, and heated again for 20 minutes. Once cool again, 2 mL of saturated NaCl was added, homogenized in a vortex(for 2 minutes), then 10 ml of n-hexane was added, homogenized again, and the samples were then kept at room temperature. The hexane-methyl ester layer was collected, transferred into a 10 mL volumetric flask, diluted with n-hexane. Furthermore, the solution was injected into the gas chromatograph, and the types of fatty acid samples were determined based on the standard solution. Fatty acid levels were analyzed descriptively.

3. Results and Discussion

Sperm motility index and viability

The sperm motility level post-electroporation is shown in Table 1, and significant differences were observed among treatments ($p<0.05$). The A100 treatment (electroporation at a DNA concentration of 100 µg mL^{-1}) had similar sperm motility index compared to control. The sperm motility index was slightly different in the A25 treatment (electroporation at a DNA concentration of 25 µg mL^{-1}). Meanwhile, no significant differences were noticed among treatments in terms of sperm viability (Figure 1). Thus, differences in DNA concentrations only affectedsperm motility, not its viability.

Table 1. Motility index and viability of electrophoresed sperm with different DNA concentration.

Treatments	Motility index (score)	Sperm viability (%)
A25	0.75 ± 0.35^{a}	$62.95\pm2,11^{a}$
A50	1.50 ± 0.71^{ab}	68.79 ± 7.80^{a}
A100	3.50 ± 0.71^{b}	71.97 ± 7.88^{a}
STP	2.50 ± 0.71^{ab}	80.10 ± 4.47^{a}
STD	1.50 ± 0.71^{ab}	71.07 ± 0.79^{a}

Description: Data are presented as mean ± SD of 3 replicates. A25 = treament with a DNA concentration of 25 µg mL^{-1} ; A50 = treament with a DNA concentration of 50 µg mL^{-1} ; A100 = treament with a DNA concentration of 100 µg mL^{-1}.STP = Control treatment without electroporation; STD = Electrphoresed control treament without DNA. The same superscript letters showed results that did not significantly differ based on Tukey's range test ($p<0.05$).sperm motility score was carried based on Guest et al. (1976).

In the present study, electrophoresed sperm viability was still similar to that of the control, which could be due to the use of *square wave* electroporation method that might possibly damage sperm cells in smaller catfish. Chen et al. (2009) reported similar results. They stated that the *square wave* method generated a high voltage in a short wave, which caused heat. Furthermore, DNA transfer could take place without killing the cell, and a good sperm wouldwell fertilize the egg. Changes in sperm movement (motility) and viability would affect sperm performance.

PCR results with electrophoresed sperm DNA demonstrated that OmΔ6FAD gene could be detected in the A100 treatment, having similar size as the control plasmid K* (529 bp). Meanwhile, no DNA bands were observed in the A25 and A50 treatments (Figure 1). K-Control did not have DNA bands from the PCR products, indicating that no contamination occurred during the PCR amplification process. Thus, A100 treatment was selected for further research.

Fertilization, hatching, and larvae survival rate

Fertilization rate (FR), hatching rate (HR), and larvae survival rate (SR) are presented in figure 2. The results showed no significant differences among treatments ($p>0.05$), confirming that electrophoration the A100 DNA concentration treatment did not affect FR, HR, and SR. These results were in accordance with Tsai (2000) founding, who reported that the HR of eggs fertilized by electrophoresed sperm was similar to that of the control sperm (without electroporation).

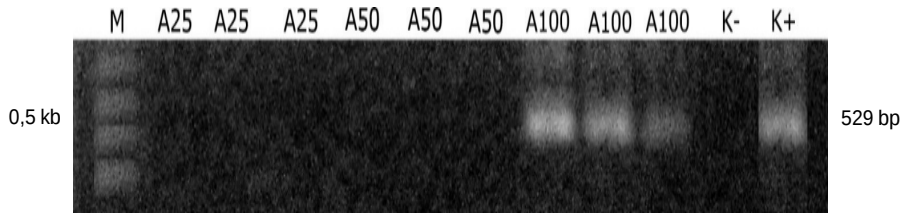

Figure 1. Electropherogramof PCR product using a specific OmΔ6FADprimer. A25 = treament with a DNA concentration of 25 µg mL^{-1} ; A50 = treament with a DNA concentration of50 µg mL^{-1} ; A100 = treament with a DNA concentration of100 µg mL^{-1}. Information: M = DNA *marker Kapa DNA ladder* (USA); K^{-} = PCR product without DNA*template*; K^{+} = PCR product with a mBP-OmΔ6FAD plasmid*template*.

Fertilization, hatching, and larvae survival rate

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Transgenic fish (F0) percentage

PCR analysis results using OmΔ6FAD as a specific primer for F0 transgenic fish identification is presented in figure 3. Based on the figure 3, it could be seen that not all the electrophoresed fish (treatment), from sample 1 up to 13, had DNA bands similar to those of the control plasmid k*(529 bp). Using β-actin as primer, all the test fish samples possessed DNA bands from the PCR product, signifying that no mistake was made during PCR and DNA extraction processes. No PCR product was observed in the negative control (without DNA *template*), showing that there was no contamination during PCR processes. Moreover, no PCR product was observed in the non-transgenic control fish treatment, stipulating that the primer used was specific for the OmΔ6FAD gene and there was no *cross-annealing*with the catfish genome DNA. Thus, electroporation treatment fish number 2, 3, 5, 7, 9, 11 and 13 were transgenic fish (F0) (figure 3).

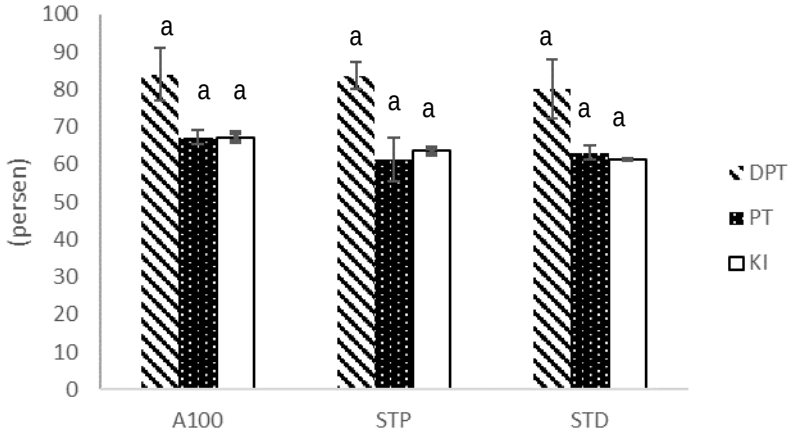

Figure 2. Fertilization rate (FR), hatching rate (HR), and larvae survival rate (SR). A100 = treament with a DNA concentration of100 µg mL^{-1} ; STP = Control treatment without electroporation; STD = Electrphoresed control treament without DNA. Letters above the same bar showed results that did not significantly differ based on Tukey's range test ($p<0.05$).

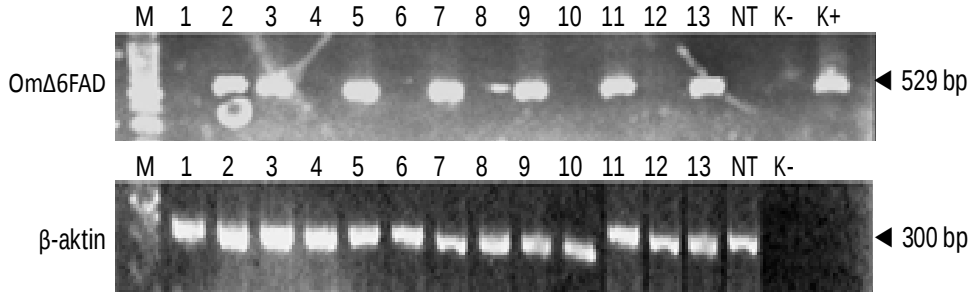

Figure 3. Electropherogramof PCR product using a specific OmΔ6FAD primer (above), and β-actin (below). Description: M = DNA *marker Kapa DNA ladder* (USA); PCR products from 1 to 13 with DNA from test fish sample. NT= PCR products with non-transgenic fish *template;* K^- = PCR product without DNA*template*; K^+ = PCR product with an mBP-OmΔ6FAD plasmid*template*. Arrowhead signs are PCR amplification products of targeted DNA.

The percentage transgenic fish founder (F0) carrying the OmΔ6FAD gene was 53,84%. In fact, F0 percentage was shown to be higher (in the current study) compared to that of the previous research, in particular, Zebra fish using the OmΔ6FAD encoding gene (44.1%) (Alimuddin *et al.*, 2005). These differences were believed to be due to dissimilarity in terms of methods and fish species. Alimuddin *et al.* (2005) reported the use of microinjection method. Furthermore, Symonds *et al.* (1994) stated that the efficacy of fastening exogenous DNA through electroporation in *Chinook* salmon is determined by the electric field, the number of electric shocks and the DNA concentration.

Fatty acids profile

The fatty acid profile of transgenic catfish (F0), non-transgenic catfish and feed worm (given feed) is shown in Table 2. In general, no differences were observed in terms of EPA and DHA contents between transgenic and non-transgenic fish. Thus, OmΔ6FADenzyme activity has not been detected yet in transgenic (F0) catfish.

Fatty acid contents in both transgenic (F0) and non-transgenic fish, especially18:2n-6, 20:4n-6, and 20:3n-3, were observed to be higher compared to that of the worm (table 2). It indicated that catfish accumulated fatty acids in their body, which was in accordance with Ackman (1982) who stated that fatty acids contained in fish is not a result of synthesis in the fish body, but of fatty acids accumulation from the food chain. In addition, DHA was detected in both transgenic (F0) and non-transgenic catfish, but not in worm, indicating that catfish had the ability to synthesis DHA. In general, freshwater fish have the ability to synthesize HUFA such as DHA, EPA and arachidonic acid(ARA; 20:4n-6) from LNA and linoleic acid (LA; 18:2n-6), since they possess the enzymes for biosynthesis including Δ6-desaturase and / or Δ5-desaturase and elongase (Tocher, 2003).

The absence of differences in terms of fatty acids in transgenic and non-transgenic fish was due to the expression variations between individual and transgenic fish (F0) that were still having mosaic behaviors since not all the F0 fish carried transgene in their tissue (Chou *et al.*, 2001). In general, transgenic fish performance is assessed in the F2 generation. Due to that fact, further research need to carried out to determine F0 transgenic fish carrying transgene (in the gamete), which will be used to produce the first generation (F1). In the first generation, all the levels have expressions with the same degree, so that there is a need to select individual with high expression for producing the second generation (F2). Therefore, fatty acid contents analysis that describes OmΔ6FAD enzyme activity could be done in second generation transgenic fish (F2).

Table 2. Fatty acids profile (%) of transgenic fish, non-transgenic fish, and feed (worms).

Fatty acids	**TG**	**NTG**	**Worms**
18:2n-6	0,25	0,15	0,11
18:3n-3	0,02	0,01	0,01
20:2n-6	0,12	0,08	0,05
20:3n-6	0,04	0,03	0,02
20:4n-6 (ARA)	0,17	0,17	0,08
20:3n-3	0,01	0,01	0,002
20:5n-3 (EPA)	**0,01**	**0,01**	**0,01**
22:6n-3 (DHA)	**0,03**	**0,05**	***Not detected***
Σ n-6	0,59	0,45	0,27
Σ n-3	0,08	0,08	0,03
Σ n-3 HUFA	0,06	0,07	0,02

Description: TG = Transgenic fish; NTG = Non-transgenic fish

4. Conclusion

The transgenic catfish founder (F0) was successfully produced using electroporation method through sperm-mediated gene transfer.The best DNA concentration for catfish sperm electroporation program is 100 µg $ml^{-1.}$ The result of fatty acid analysis showed that EPA an DHA contents of F0 transgenic fish and non-transgenic fish were simliar.

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